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Cat eye chromosome duplication breakpoints associated with the human cat eye syndrome cluster in two regions that correspond to deletion breakpoints of the CATCH22 syndrome

by

Kerry Ellen M^cTaggart ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

Molecular Biology and Genetics

Department of Biological Sciences

Edmonton, Alberta
Fall, 1997

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "Cat eye chromosome duplication breakpoints associated with the human cat eye syndrome cluster in two regions that correspond to deletion breakpoints of the CATCH22 syndrome", submitted by Kerry Ellen McTaggart in partial fulfillment of the requirements for the degree of Master of Science in Molecular Biology and Genetics.

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Abstract

CES is a rare, phenotypically variable human disorder resulting from a duplication of 22pter-22q11.2, typically in the form of a supernumerary dicentric bisatellited cat eye chromosome, or CEC. CECs are divided into two groups based on the location of the two breakpoints. Through fluorescence *in situ* hybridization and quantitative dosage analysis, I have more specifically defined the locations of these breakpoints. I have demonstrated that the breakpoints of the smaller type I symmetrical CECs occur in a 450-650kb interval corresponding to the published proximal deletion breakpoints associated with a second syndrome, CATCH22. The larger type II CEC duplications localize to the same interval as the CATCH22 syndrome distal deletion breakpoints. The clustering of rearrangement breakpoints in 22q11.2 may be the result of the presence of repetitive sequences, as has been demonstrated in conditions involving rearrangements of other chromosomes.

Acknowledgements

First and foremost, I would like to thank my supervisor, Heather McDermid for her patience, wisdom, and assistance above and beyond the call of duty over the last three years.

Alberta Heritage Foundation for Medical Research for financial support

Karen Romanyk, Dana Shkolny, and Angela Johnson for superb technical assistance and for serving on the "sanity maintenance" committee

The running gang and the "Roaches"

The McDermid lab for companionship and assistance in the lab

John Locke, Mark Hicks, Andrew Simmonds, and Jeff Hoyem for patiently assisting a computer illiterate

The Philly group- in particular, Marcia Budarf for supplying "key" clones for the CEC breakpoint analysis and Barry Barnoski for over-the-phone and over-the-fax FISH technical advice

John Groffen at the Children's Hospital Los Angeles for supplying plasmid CRKL

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Abbreviations

ARRC22= Anchored Repeat Rearrangements of Chromosome 22
AS= Angelman syndrome
ASD= atrial septal defect
BL= Burkitt's lymphoma
BSA= bovine serum albumin
BWIS= Baltimore-Washington infant study
CATCH22= Conotruncal heart anomalies, Abnormal facies, Thymic hypoplasia/aplasia, Cleft palate, and Hypocalcemia associated with a microdeletion in 22q11.2
CEC= cat eye chromosome
CEPH= Centre d'Etude du Polymorphisme Humain
CES= cat eye syndrome/ Schmid-Fraccaro syndrome
CESCR= cat eye syndrome critical region
CHARGE= Coloboma, Heart anomaly, Atresia choanal, Retardation, Genital anomalies, Ear anomalies
CHD= congenital heart defect
CLTCL= clathrin heavy chain-like gene
cM= centimorgans
CM #= a patient designation indicating the presence of a typical supernumerary CEC
CML= chronic myelogenous leukemia
CMT1A= Charcot-Marie-Tooth disease type 1A
COMT= catechol-o-methyltransferase
CRKL= *crk* -like gene
CTAB= cetyltrimethylammonium bromide
CTAFS= conotruncal anomaly face syndrome / Takao syndrome
CTP= citrate transporter protein
CVS= chorionic villus sampling
DGCR= DiGeorge critical region
DGS= DiGeorge syndrome
DMEM= Dulbecco's modifed Eagle medium
DMSO= dimethyl sulfoxide
DNA= deoxyribonucleic acid
DTAB= dodecyltrimethylammonium bromide
DVL-22= *dishevelled* gene
EBV= Epstein-Barr virus
EDTA= ethylenediaminetetra-acetic acid disodium salt
ES= Ewing's sarcoma
FBS= fetal bovine serum

FITC= fluorescein isothiocyanate
FISH= fluorescence *in situ* hybridization
GDB= Genome DataBase
GGT= gamma glutamyl transferase
GSCL= *goosecoid* -like gene
HBSS= Hank's balanced salt solution
HCF= heparin cofactor
HEPES= (N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid])
HNPP= hereditary neuropathy with liability to pressure palsies
IAA= interrupted aortic arch
Ig= immunoglobulin
IUGR= intrauterine growth retardation
kb= kilobases of DNA
LB= Luria-Bertani
LINE= long interspersed repetitive element
Mb= Megabase of DNA
MEM= minimum essential medium
MITE= mariner transposon-like element
MR= mental retardation
OLB= oligo labeling buffer
OMIM= Online Mendelian Inheritance in Man
PCR= polymerase chain reaction
PDA= patent ductus arteriosus
PFGE= pulsed field gel electrophoresis
PMP22= gene for peripheral myelin protein 22
PWS= Prader-Willi syndrome
r(22)= ring chromosome 22
RAA= right aortic arch
RBC= red blood cell
RFLP= restriction fragment length polymorphism
SDS= sodium dodecyl sulfate
SRO= smallest region of overlap
STS= steroid sulfatase
TAPVR/TAPVD= total anomalous pulmonary venous return/drainage
TOF= tetralogy of Fallot
UPD= uniparental disomy
VCFS= velocardiofacial syndrome/ Shprintzen syndrome
VNTR= variable nucleotide tandem repeat
VSD= ventricular septal defect
YAC= yeast artificial chromosome

INTRODUCTION

• Chromosome 22

Chromosome 22 (Figure 1) is the second smallest autosome, accounting for 1.7% to 1.8% of the total human genome and representing an estimated 52 to 56 Mb of DNA (Kaplan et al., 1987; Morton 1991). Chromosome 22 is one of five pairs of acrocentric chromosomes, the others being chromosomes 13, 14, 15, and 21. Characteristic of the acrocentrics are disproportionately small p-arms (p="petit") that share similar organization. These p-arms consist mainly of repetitive DNA and are divided into three regions. The pericentromeric, or p11 region consists primarily of repetitive sequence families based on a 5 base pair core repeat unit (Frommer et al., 1982; Prosser et al., 1986). Distal to the p11 region is a secondary constriction, or "stalk", designated p12. The secondary constriction is the location of the nucleolar organizing regions (NORs), consisting of a 44 kb segment of tandemly arranged genes encoding the 5.8S, 18S and 28S ribosomal RNA (rRNA) molecules (Schmickel 1973; Worton et al., 1988; Henderson et al., 1972). The rRNA genes are the only genes known to be present on the acrocentric p-arms. The most telomeric segment of the p-arm is p13 and is a satellite, or mass of DNA. The satellite is the most distinguishing feature of the acrocentrics as it is detectable cytogenetically and can have useful size heteromorphisms (chromosomal polymorphisms), enabling the differentiation of homologs of a chromosome pair in an individual.

The q-arms of the acrocentrics, in contrast to the p-arms, are different from one another. The q-arm of chromosome 22 represents 1.4% of the total length of the autosomal genome and 43Mb of DNA (Patau, unpublished from Therman & Susman 1993; Morton 1991) It is divided into three bands at the 400-band level: a G-light q11 band comprising approximately 30% of 22q, a G-dark q12 band accounting for approximately 20%, and another G-light band q13 accounting for the remaining 50%. The chromosome can be further subdivided into 14 subbands at the 850-band level (Figure 1). G-light bands are generally early replicating and contain

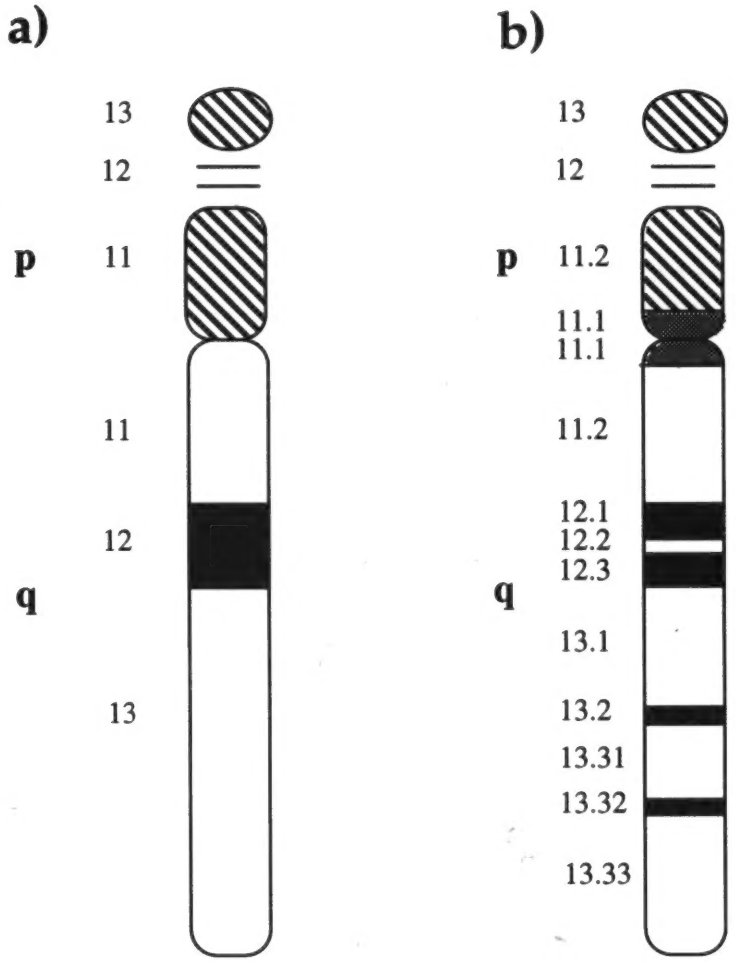


Figure 1. Ideograms of chromosome 22 at the (a) 400- and (b) 850-band levels (modified from Mears 1995).

relatively unmethylated, GC rich sequences (Sentis et al., 1993). G-light bands contain the highest density of CpG islands, which are stretches of undermethylated GC-rich sequences associated with the 5′ ends of 50% to 60% of the genes in the mammalian genome (a conservative estimate) (Craig & Bickmore 1994; Fields et al., 1994). This information on CpG island density, as well as the observation that 80% of the greater than 1000 human genes that had been mapped by 1993 were situated in G-light bands (Craig & Bickmore 1993) suggested that CpG island density was indicative of gene density. The mainly G-light (and thus gene-rich) composition of the long arm of chromosome 22, in addition to its small size, make it an attractive chromosome to characterize. Both genetic and physical mapping efforts are currently in progress, with the goal that chromosome 22 will be the first chromosome sequenced in its entirety.

Early mapping efforts attempted to determine the genetic size or length of the chromosomes and produce a mapped series of probes. Dumanski et al (1991) produced one of the first detailed genetic maps, placing 29 markers representing 22 loci over the q-arm, a genetic distance of approximately 110 cM. Frazer et al (1992) constructed a radiation hybrid map, and Gyapay et al (1994) and Buetow et al (1993) constructed genetic maps based on STS markers.

Physical mapping began with the construction of chromosome 22-specific somatic cell hybrid panels (Budarf et al., 1991; Delattre et al., 1991). Somatic cell hybrids are human-rodent hybrid cell lines, each one containing one to a few human chromosomes or fragments of chromosomes. The human fragments in the above panels were obtained from both naturally occurring translocations and cell culture induced rearrangements involving chromosome 22. The hybrid cell lines are used to generally localize probes, or markers, by dividing the chromosome through rearrangement breakpoints into intervals called "bins". The most recent panel of Budarf et al (1996) subdivides the chromosome into 24 bins, enabling greater than 300 probes to be mapped to these intervals. The order of the probes within the intervals cannot be determined using this mapping technique, however it does facilitate the integration of physical and genetic maps.

Long-range physical maps, constructed from pulsed field gel electrophoresis (PFGE) analysis, have been used to assign order and actual distances between mapped probes (McDermid et al., 1993; McDermid et al., 1996). YAC contigs have been constructed to order probes as well as clone the region. There are two major mapping efforts on chromosome 22; one in Philadelphia and the other at the Sanger center inEngland. As part of the Philadelphia effort, we have produced a YAC contig of the most proximal 3.6Mb of 22q11.2 (McDermid et al., 1996).

In addition to the large number of potential genes on 22q and its small size, another reason for its attractiveness to researchers are the large number of chromosomal rearrangements associated with many congenital and acquired conditions. Characterization of the chromosome therefore affords an opportunity to study these genes' roles in development as well as causes of chromosome rearrangement.

• Acquired conditions resulting from 22q rearrangements

There are several well characterized examples of acquired conditions resulting from recurrent balanced translocations involving chromosome 22. In all three of the malignant conditions summarized in Table 1, malignancy is a result of the translocation of a gene into a new chromosomal location, the effect of which is either the production of a chimeric protein, or the alteration of gene expression.

Table 1. Acquired malignant conditions resulting from recurrent balanced translocations involving chromosome 22.

Condition		Break-point	Affected Locus	Break-point	Affected Locus	% of Cases	Result of Translocation
Burkitt's Lymphoma (BL)[1-4]	B-cell tumor of the jaw	22q11 2q11 14q32	**lambda light chain** **kappa light chain** **IgH locus**	8q24	***myc*** (proto-oncogene; nuclear transcription factor)	15% 5% 80%	Translocation of *myc* adjacent to Ig locus control elements resulting in continued expression and prevention of B- and T-cell differentiation
Chronic Myelogenous Leukemia (CML)[5-7]	bone marrow (myeloid) cancer	22q11	***bcr***	9q34	***abl*** (cytoplasmic tyrosine kinase)	90-95%	Results in a *bcr-abl* hybrid oncogene with increased tyrosine kinase activity
Ewing Sarcoma (ES)[8-13]	"primitive" small round cell tumor	22q12	**EWS** (possible RNA binding protein)	11q24 21q21	**FLI** **ERG** (ETS transcription factors)	most 5-10%	Chimeric product is a stronger transcriptional activator

[1]Thompson et al., 1991; [2]Berger et al., 1979; [3]Zech et al., 1976; [4]Lewin 1994

[5]Rowley 1973; [6]Shtivelman et al 1983; [7]Konopka et al 1985

[8]Denny 1996; [9]Aurius et al., 1983; [10]Sorensen et al., 1994; [11]Delattre et al., 1992; [12]Zucman et al., 1993; [13]May et al., 1993

• Congenital conditions associated with duplications of chromosome 22q

In addition to the acquired conditions associated with aberrations of chromosome 22, there are numerous congenital conditions. Some of these are associated with duplication of a portion or all of chromosome 22 and others with a partial deletion. There are three conditions associated with the presence of a duplication; trisomy 22, the recurrent 11q23;22q11 translocation derivative 22 syndrome [der(22)t(11;22)(q23;q11)], and cat eye syndrome (CES). These conditions are duplicated for varying lengths of 22q, although do have a shared region of duplication, suggesting that these conditions may share some phenotypic features. The focus of this thesis is the smallest of these duplications, which results in CES.

• Cat eye syndrome (CES)

• Features of CES

Cat eye syndrome (OMIM#115470) is associated with a variety of congenital defects summarized in Table 2. The phenotype of CES is highly variable in both the features present and their severity. Most of the features are non-specific, meaning they exist as isolated anomalies in chromosomally normal individuals as well, and as common features in other syndromes or disorders. What makes the features of CES characteristic of this syndrome is the association of these particular features.

CES derives its name from the iris coloboma, even though it is not present in all CES individuals. Coloboma results from the incomplete closure of the optic fissure during the sixth to eighth weeks of development (Moore & Persaud, 1993) and in addition to the iris, it can also affect the retina and/or choroid. Haab in 1878 first described an association between coloboma and anal atresia, which was later named CES (Gerald et al., 1968). Anal atresia is due the absence of an anal canal. Imperforate anus, or membranous anal atresia, is a result of the persistence of the cloacal membrane which usually ruptures at the end of

Table 2. Summary of CES features and estimations of frequency.

Phenotypic Features	Frequency
Preauricular pits and/or skin tags	75%
Ear anomalies	43%
Hypertelorism	40%
Downslanting palpebral fissures	55%
Epicanthal folds	19%
Ocular coloboma	52%
Other ocular anomalies	36%
Broad flat nasal bridge	14%
Micrognathia	30%
Congenital heart defects	44%
Anal anomalies	66%
Renal anomalies	38%
Genital anomalies	32%
Skeletal anomalies	27%

References: Zellweger et al., 1962; Schachenmann et al., 1965; Ishmael & Laurence 1965 Neu et al., 1970; Pfeiffer et al., 1970; Weber et al., 1970; Darby & Hughes, 1971; Krmpotik et al., 1971; Gerald et al., 1968; Gerald et al., 1972; Fryns et al., 1972; Bühler et al., 1972; Newton et al., 1972 Cory & Jamison, 1974; Niermeijer et al., 1976; Toomey et al., 1977; Weleber et al., 1977; Garlinger et al., 1977; Petit et al., 1980; Smith et al., 1981; Schinzel et al., 1981a; Guanti 1981; Weilong et al., 1982; Chemke et al., 1983; Wilson et al., 1984; Rosenfeld et al., 1984; Mahboubi & Templeton 1984; Buckton et al., 1985; Reiss et al., 1985; Gabarron et al., 1985; Hoo et al., 1986; Ing et al., 1987; Magenis et al., 1988; Luleci et al., 1989; Ward et al., 1989; Liehr et al., 1992; Cullen et al., 1993; Urioste et al., 1994; Mears et al., 1994; Hough et al., 1995; Lindsay et al., 1995; Knoll et al., 1995; Mears et al., 1995; Prasher et al., 1995; McTaggart et al., 1996.

the eighth week of development (Moore & Persaud, 1993). Anal atresia is often associated with the presence of a fistula, or an abnormal passageway or tube connecting two structures. Fistulas can connect the rectum to various other structures such as the vagina (rectovaginal), urethra (rectourethral), bladder (rectovesical), and peritoneal cavity (rectoperitoneal). Displacement of the anus and anal stenosis, or narrowing of the anal canal, have also been described in individuals with CES.

Also associated with these two cardinal features are congenital heart defects (CHD), the most common of which are total anomalous pulmonary venous drainage/return (TAPVD or TAPVR), tetralogy of Fallot (TOF), and ventricular septal defects (VSD). Atrial septal defects (ASDs), interrupted aortic arch (IAA), patent ductus arteriosus (PDA), and coarctation of the aorta have also been reported.

TAPVR is a cyanotic (deficient oxygenation of the blood) type of heart defect, in which the pulmonary veins returning from the lungs with oxygenated blood fail to connect with the left atrium and instead connect directly to the right atrium or one of the systemic veins draining into the right atrium (Neill 1956; Rowe et al., 1981). Aberrant connections between the left and right ventricles in these patients enables limited survival even though the blood being pumped from the left ventricle to the body is less oxygenated than normal. This defect is usually surgically corrected early in life. In utero, this defect is not detrimental because the fetal circulatory system differs from that of a newborn. The lungs are not yet functional and blood is oxygenated by the placenta. Oxygenated blood from the placenta enters the right atrium through the inferior vena cava, which in adults normally returns deoxygenated blood from the lower body which then gets pumped to the lungs by the right ventricle for oxygenation. The positioning of the inferior vena cava is such that when blood enters the right atrium, it passes almost entirely into the left atrium via the foramen ovale, or "hole" between the right and left atria that exists in the fetus. This highly oxygenated blood then passes into the left ventricle which then pumps it throughout the body. Thus the aberrant connections of the vessels returning from the lungs with the right atrium are of no

consequence. Newborn circulation however is compromised. When the lungs become functional, oxygenated blood returns via the pulmonary veins, which in TAPVR, connect to the wrong side of the heart.

A review of the literature shows that TAPVR accounts for slightly less than half of the heart defects in individuals with CES. It is a relatively serious defect and is rare in the general population, accounting for approximately 1.5% of cases of cardiovascular malformations as estimated in a Baltimore-Washington Infant Study (BWIS) conducted from 1981 to 1987 (Correa-Villasenor et al., 1991). Results from this study estimated that about half of infants with this defect survived to one year of age, suggesting that TAPVR was a major cause of lethality in CES. Current surgical intervention is more successful and the survival rate of infants born with TAPVR is now higher than this. Both the rarity of this heart defect in the general population and its frequency of occurrence in individuals with CES suggest that TAPVR is a cardinal feature of this disorder.

TOF is also a cyanotic type of heart defect, however is not as serious as TAPVR. TOF is a more common defect than TAPVR in the general population, with an incidence of 7.56% in newborns (Ferencz et al., 1989). TOF is a combination of the four defects listed below:

- Pulmonary stenosis: narrowing of the pulmonary artery that leaves the right ventricle, carrying blood to the lungs for oxygenation
- Ventricular septal defect (VSD): a hole which enables "mixing" of the blood between the two ventricles, resulting in the reduction in oxygenation of the blood distributed to the body
- Over-riding aorta: the aorta is positioned so that it covers the VSD, thus enabling blood from both ventricles (oxygenated and deoxygenated) to enter and be distributed throughout the body
- Hypertrophy of the right ventricle: right ventricle is larger than normal

VSDs are the most common heart defect among infants, accounting for more than one-quarter of CHDs among newborns. 80% of the cases occur as isolated anomalies. VSDs are also the most common CHD among abortuses and stillborns (Hoffman 1995).

Other organ systems may also be affected in CES. For example, renal defects are common and include hypoplasia of the kidneys (underdeveloped kidneys), unilateral kidney agenesis (absent kidney), recurrent urinary infections, hydronephrosis (urine flow obstruction), and vesicoureteral reflux (backward flow of urine from the bladder to ureter). Skeletal [scoliosis (crooked spine), malformed/fused vertebrae, kyphosis (humpback/flexion of the spine), supernumerary/extra ribs, clinodactyly (finger deflection)] and genital systems [hypoplasia of external genitalia, cryptorchidism (undescended testes), hypospadias (urethral opening on underside of penis), and rudimentary (incompletely developed) internal genitalia] may also be seen, although at a lower frequency than renal defects. Gastrointestinal abnormalities such as malrotation of the gut, and Hirschsprung's disease (congenital megacolon, or absence of ganglion cells from varying lengths of segments of the colon) have also been reported (Mahboubi & Templeton, 1984; Mears et al., 1994; Ward et al., 1989; Guanti et al., 1981; our unpublished data).

Characteristic CES facial features include preauricular skin tags and pits formed by the abnormal development of the auricle (external portion of the ear), hypertelorism (widely set eyes), downslanting palpebral fissures, and micrognathia (small jaw). Less frequently associated features include palatal anomalies (cleft lip and/or palate, high arched palate, and bifid uvula), broad flat nasal bridge, epicanthal folds, malformed posteriorly rotated ears, low set ears, cheek skin tags or pits, and a variety of ocular anomalies such as microphthalmia (small eyes), strabismus (crossed eyes), Duane anomaly (neurological disorder which results in difficulties in gaze adduction) and nystagmus (jerky eye movements).

Mental retardation is also a feature of this syndrome. Individuals with CES typically have mild to moderate mental retardation, although there have been reports of individuals within the normal range. This

feature is not listed in Table 2 due to the difficulties posed in the diagnosis of young patients.

It is very difficult to assign frequencies to each of the features listed in Table 2 for several reasons. Cases are examined by different clinicians, introducing variability, especially for those characteristics which require more subjectivity in their ascertainment. Facial features for example, vary tremendously in the normal population, making the distinction of normal from abnormal problematic. As well, not all features may be reported or even investigated fully (for example ultrasound to detect kidney hypoplasia or aplasia, or a full ophthalmologic examination to detect retinal and choroidal coloboma). Thus, the lack of report of a particular feature does not necessarily indicate it is absent.

There is also the problem of underdiagnosis of CES as a result of the tremendous phenotypic variability in terms of both the presence and severity of features. It is highly probable that a small number of CES individuals never get diagnosed because their features are not serious enough to ever come to the attention of a physician (for example, absence of a serious heart defect and anal atresia). Many of the features of CES occur as isolated anomalies. For instance, a patient with borderline hypertelorism, a preauricular pit, mild micrognathia, an ASD which may not be diagnosed until adulthood, and kidney hypoplasia detectable only upon ultrasound, might not be recognized as CES. Thus, the frequencies calculated for the more obvious or more life threatening anomalies such as TAPVR, anal atresia or iris coloboma, are likely to be overestimates due to the bias associated with CES diagnosis.

- History and Etiology of CES

In 1965, Schachenmann et al reported the association of a supernumerary small submetacentric chromosome in individuals with ocular coloboma, anal atresia, and similar facial appearance. This was the first suggestion that this phenotype was the result of a chromosomal abnormality. Cytogenetics revealed the presence of satellites on these supernumerary "marker" chromosomes (Schachenmann et al., 1965).

Thus the chromosomal origin of these supernumerary chromosomes was narrowed down to chromosomes 13, 14, 15, 21, or 22.

Following the 1965 report by Schachenmann, other cases were described that had similar phenotypes associated with an acrocentric or submetacentric chromosome slightly smaller in size than a normal G-group (chromosomes 21 or 22) chromosome. Other than the fact that it had satellites and therefore of acrocentric origin, it was not known from which of the 5 pairs it was derived. Observations that it was late labeling by autoradiography (chromosomes 13, 14, or 22), and that the clinical manifestation shared few features with trisomy 21/Downs syndrome or trisomy 13, suggested its origin from either chromosome 14 or 22 (Pfeiffer et al., 1970; Weber et al., 1970). Others argued it was a partially deleted D-group chromosome (chromosomes 13, 14, or 15) because of the overlap of phenotypic features such as coloboma which is seen in trisomy 13 (Ishmael & Laurence 1965; Cory & Jamison 1974; Zellweger et al., 1962; Krmpotik et al., 1971; Rosenfeld et al., 1984; Guanti et al., 1981).

Even with the advent of chromosomal banding in the early 1970's, the origin was difficult to determine due to the small size of the chromosome as well as the fact that it was mostly p-arm and centromeric heterochromatin with no distinct banding pattern. Satellite and NOR heteromorphisms helped in some cases to identify the chromosome of origin as chromosome 22 (Toomey et al., 1977). However, as Guanti et al (1981) and Petit et al (1980) state, a crossover may have occurred between chromosome 13q and 22p or proximal 22q. This would result in a chromosome 13 q-arm with a chromosome 22 p-arm, making p-arm heteromorphisms not definitive as a measure of chromosomal origin. Despite this however, the supernumerary marker chromosome was generally believed to be at least partially derived from chromosome 22 because of autoradiographic labeling studies and the partial phenotypic overlap of CES with trisomy 22 (Smith et al., 1981; see below). Several reports in the literature postulated that the supernumerary chromosome was formed from two acrocentric chromosomes, at least one of which was chromosome 22 (Schinzel et al., 1981a; Toomey et al., 1977; Weleber et al., 1977).

The origin of the extra chromosome was confirmed as entirely chromosome 22 using the probe p22/34, located in proximal 22q. Using quantitative analysis of Southern blots, M'Dermid et al (1986) demonstrated the presence of four copies of this probe in all the individuals tested. Thus the "CEC", or cat eye chromosome (Bühler et al., 1972), was an inverted duplication of 22p and the proximal portion of 22q resulting in four copies of this region.

- CEC Stability

The CEC has a dicentric bisatellited structure with only one primary constriction, or functional centromere, indicating stability achieved through an unknown process. Normally, during cell division, spindle attachment and subsequent segregation would result in breakage of a dicentric structure. Despite the dicentric structure of the CEC however, it can be very stable. Evidence in support of this is the existence of non-mosaic individuals as well as cases of CEC transmission to subsequent generations (Schachenmann et al., 1965; Darby & Hughes, 1971; Krmpotik et al., 1971; Gerald et al., 1972; Chemke et al., 1983; Luleci et al., 1989; Cullen et al., 1993). It is believed that this stability is a result of either centromere inactivation or cooperation. These processes are likely dependent on how far apart the centromeres are. Support for these processes are the tremendous number of Robertsonian translocations that are structurally dicentric but functionally monocentric (John & Freeman 1975; Sears & Camera 1952; Therman et al., 1974, 1986; Hsu et al., 1975).

Results of a study examining dicentric iso X chromosomes suggested that the distance between centromeres determined stability. Those chromosomes with centromeres greater than 20Mb or less than 3.5Mb apart were more stable than those with centromeres separated by 3.5Mb to 20Mb which were functionally dicentric (Sullivan et al., 1996). The more stable dicentrics were functionally monocentric as a result of either centromere cooperation or inactivation. The choice of which of these two processes occurs may be distance dependent. In the cases of the CECs, behavior as a functional monocentric is thus likely a result of

centromere inactivation due to the amount of genetic material between the two centromeres and the cytogenetic observations of a dark G-band on the q-arm believed to be the inactivated centromere. Those CECs demonstrating instability may be a result of the presence of two active centromeres.

Some evidence exists in support of instability of some CECs, resulting in mosaicism due to loss of the CEC. The presence of mosaicism is usually associated with a milder phenotype (Urioste et al., 1994; Cullen et al., 1993). The degree of mosaicism may change over time and different levels of mosaicism may exist in different tissues tested. Cases of familial transmission in which a proband has received the chromosome from a parent and thus started out as a nonmosiac but is mosaic at birth are examples demonstrating the instability of these chromosomes. Often members of a family will show different levels of mosaicism (Urioste et al., 1994; Cullen et al., 1993).

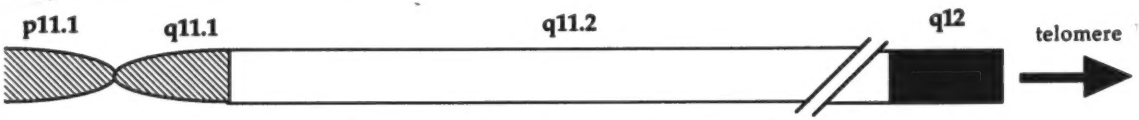
Probably the most striking examples of instability of dicentric chromosomes are those in which different cells of the same individual contain different forms of the original chromosome. Van Dyke et al (1977) reported an individual with three different cell lines. One of the cell lines was normal, another contained a dicentric marker chromosome, and the last had in addition to the marker, a smaller derivative that was also dicentric and bisatellited. Urioste et al (1994) reported a family in which a mother and her three daughters were not only mosaic for a supernumerary chromosome but for the type of chromosome. Bisatellited chromosomes with two or a single NOR were observed, as well as monosatellited chromosomes, ring chromosomes and fragments of various sizes, were found in different cells of all three individuals (Urioste et al., 1994).

• CEC Duplication Breakpoints

The formation of the CEC involved two breakpoints. Mears et al (1994) and Mears (1995) localized the breakpoints of twelve CECs through RFLP and quantitative dosage analysis. A summary of those results is

given in Figure 2. The patients are listed CM01 through CM12 and the copy number of each locus is given. From the figure it is evident that there are two classes of CECs. The smaller type I CECs are relatively symmetrical with both breakpoints involved in their formation occurring in the 1Mb interval flanked by loci D22S181 and D22S36 (Mears et al., 1994). These CECs encompass a region of proximal 22q approximately 3.6Mb in size (M^{c}Dermid et al., 1996). The larger type II CECs of individuals CM02 and CM03 were asymmetrical with the proximal breakpoint located within this same 1Mb interval. The distal breakpoints of CM02 and CM03 and both breakpoints of CM01 were located somewhere beyond D22S75 (Mears et al., 1994). Hough et al (1995) demonstrated that the breakpoints of the CEC of patient CM01 were proximal to the immunoglobulin region. It was an attractive possibility that the breakpoints had occurred close together in some rearrangement "hotspot".

Figure 2. Breakpoint characterization of CECs in CES patients from Mears et al (1994) and Mears (1995). Given below the partial idiogram of chromosome 22 in table form are the copy numbers for four loci from quantitative dosage, RFLP, and FISH analysis for twelve CES patients possessing CECs. Arrows represent the locations of the breakpoints involved in the CEC formation. Above the table are the approximate physical distances between these loci, determined from PFGE analysis (McDermid et al., 1996; Mears et al., 1995).



	D22S57	D22S181	D22S36	D22S75
CM01	4	NI	4	4
CM02	4	NI	3	3
CM03	4	4	3	3
CM04	4	4	3*	2
CM05	4	4	2	2
CM06	4	4	2	2
CM07	4	4	2	2
CM08	4	4	2	2
CM09	4	4†	2	2
CM10	4	4†	2	2
CM11	4	4	3*	2
CM12	4	4	2	2

4† copy number determined by FISH

3* extra copy is the result of an interstitial duplication

NI= not informative

• Other Duplications Associated with CES

The dicentric bisatellited CEC is the most common form of duplication in CES. Other forms that the duplication may take include interstitial duplications (Reiss et al., 1985; Knoll et al., 1995), and supernumerary ring 22 chromosomes (El-Shanti et al., 1993; Mears et al., 1995; Ohashi et al., 1993). There are also reports of individuals with duplications of 22q associated with unbalanced translocations (Garlinger et al., 1977; Niermeijer et al., 1976; Bühler et al., 1972/Kosztolányi & Bühler 1985) as well as individuals with a CES-like phenotype but no apparent duplication (Neu et al., 1970; Franklin & Parslow, 1972; our unpublished cases).

CES and Interstitial Duplications

Individuals with the interstitial duplications are duplicated for varying lengths of proximal 22q. Two cases classified as CES are known (Reiss et al., 1985; Knoll et al., 1995). Phenotypic comparison to CES individuals with four copies of proximal 22q has indicated that three copies is sufficient for manifestation of all of the cardinal features of CES. Neither case had anal atresia, but the sample size is too small to conclude if this is significant. The absence of ocular coloboma in individuals with three copies of proximal 22q in the der(22)t(11;22) syndrome (see below) lead to some speculation that four copies were required for ocular coloboma, however, the presence of iris, retina and choroidal coloboma in the patient described in Reiss et al (1985) has excluded this hypothesis. The three copy individuals appear to be no more mildly affected than four copy individuals, however it is difficult to make any conclusions based on two patients.

CES and Unbalanced Translocations

There are several reports of individuals duplicated for proximal 22q as a result of an unbalanced translocation. These individuals have three copies of proximal 22q, however they also have a duplication for a portion of another chromosome, making analysis of phenotype difficult. Bühler et al (1972) report two individuals with such a chromosome, both demonstrating a CES phenotype in addition to other features not normally

reported. In another case, an individual with a derivative chromosome had a normal phenotype, indicating three copies does not always result in CES (Brøndum-Nielsen 1991).

CES and Apparently Normal Chromosomes

There are some early reports of individuals with CES-like phenotypes without the typical CEC (Neu et al., 1970; Franklin & Parslow, 1972). Cytogenetic analysis did not detect the presence of interstitial duplications, although it is plausible that small duplications or translocations may not have been detected. Prior to 1995, our lab had done some preliminary analysis on similar patients using quantitative dosage analysis with probes in the proximal 22q region and did not detect any duplications. It is entirely possible that these patients represent another syndrome such as CHARGE and Townes-Brock, both of which share some features with CES. Alternatively, the duplication may be undetectable with the resolution of probes that had been employed. Further analysis of these patients utilizing the probes available to us now may be warranted.

CES and Supernumerary Ring Chromosomes

A ring chromosome has a circular structure resulting from the breakage of both ends of a chromosome and then circularization through fusion of the ends (McClintock 1938; Lejeune 1968; Kosztolányi 1987). Ring chromosomes are usually present in individuals with 46 chromosomes, and therefore represent a deletion. Most ring chromosomes are unstable due to the mechanical forces incurred during cell division, which may result in mosaicism for monosomy, the presence of two rings per cell, a double ring, or fragments of the ring chromosome. During embryogenesis, the cells probably contain a mixture of duplications and deletions. This leads to an unpredictable phenotype typical of all ring chromosomes. It is therefore a mosaic condition rather than a straightforward deletion. The phenotype is not the same as for a deletion of the equivalent region- rings usually have severe mental retardation and little else. Coté et al (1981) described the phenotype as "ring syndrome".

The presence of a supernumerary ring chromosome, which represents a duplication, is a rare occurrence. There have been only two

reports of a supernumerary ring chromosome 22 [r(22)], and both were associated with CES (El-Shanti et al., 1993; Ohashi et al., 1993). In Mears et al (1995), a previously reported supernumerary r(22) present in three generations was characterized. Familial inheritance is unusual considering the instability normally associated with ring chromosomes. In the grandfather (CM13) and father (CM14), the presence of the ring was associated with a normal phenotype, however the proband (CM15) in the third generation had the full CES phenotype. The ring chromosome in this individual had doubled in size, likely as a result of a single sister chromatid exchange. It contains two centromeres and two copies of the region present in the r(22) in the previous two generations (Mears 1995). The normal phenotype in CM13 and CM14, again indicate that three copies of the CESCR can be associated with a normal phenotype.

Characterization of the second case of a supernumerary r(22) chromosome associated with CES has not been completed, although preliminary results by Mears indicated this r(22) is larger than the one reported by El-Shanti et al (1993).

- Definition of the CES Critical Region (CESCR)

An important component to the study of CES was to define the critical region in order to facilitate the search for genes. The CESCR is the region of proximal 22q required to be duplicated to result in the CES phenotype. Definition of the current CESCR shown in Figure 3 was accomplished by phenotypic mapping in conjunction with molecular characterization of the CES duplications.

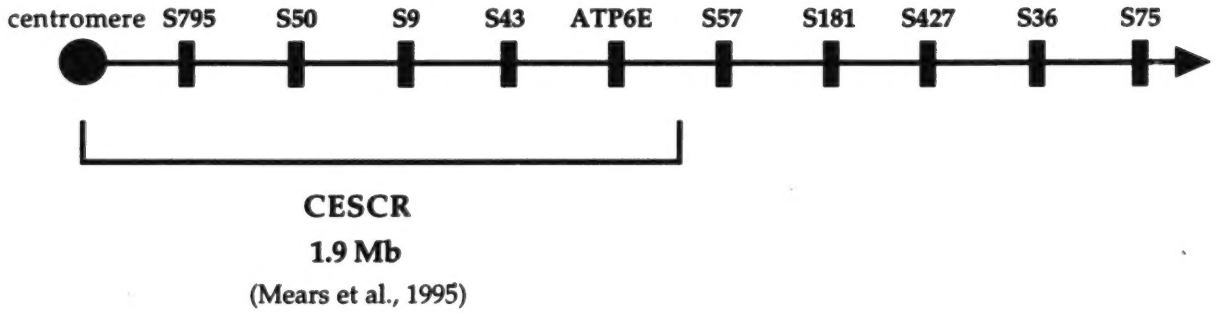
Definition of the Distal Boundary

The distal boundary of the CESCR was defined by Mears et al (1995) using the patient (CM15) with the full spectrum of CES features and a supernumerary ring 22 chromosome. This individual had a duplication with a more proximal breakpoint than those CES patients already examined, placing the distal boundary of the CESCR between loci D22S57 and ATP6E, thus narrowing the critical region to 2Mb (Mears et al., 1995).

Definition of the proximal boundary

The existence of CES individuals with interstitial duplications in proximal 22q excluded 22p from the CESCR (Reiss et al., 1985; Knoll et al., 1995). This is not surprising considering the repetitive and redundant nature of the acrocentric p-arms, evidenced by unaffected individuals with 22q;22q Robertsonian translocations who have lost both p-arms (Farah et al., 1975; Maeda et al., 1976; Lewis & Ridler, 1977; Mameli et al., 1978; Palmer et al., 1980; Kirkels et al., 1980; Schinzel et al., 1994a). Characterization of the proximal breakpoints of one interstitial duplication (Knoll et al., 1995) enabled the provisional definition of the proximal boundary of the CESCR for the features present in this patient (TAPVR, hypertelorism, preauricular pits, downslanting palpebral fissures, epicanthal folds, renal and genital defects, but not anal atresia or coloboma) to between D22S795 (N63) and D22S543, reducing the CESCR interval to approximately 1Mb in size (Mears 1995; Mears et al., in preparation). Searching for candidate genes in this region is currently in progress.

Figure 3. The CESCR in proximal 22q11.2. The minimal region required to be duplicated to result in the full CES phenotype from Mears et al (1995). This map is not drawn to scale. Loci starting with an "S" should be preceeded by "D22".



• **Der(22)t(11;22)(q23;q11) syndrome**

The most common recurrent non-Robertsonian translocation detected in man is t(11;22)(q23;q11) (Zackai & Emanuel 1980; Iselius et al., 1983; Fraccaro et al., 1980). The only viable unbalanced offspring of this translocation carrier has the karyotype 47,X_t(11;22)(q23;q11)der(22), and thus has a duplication of 11q23 to 11qter and 22pter to 22q11. The only translocation chromosome these individuals possess is the derivative (der) chromosome 22 which possesses the centromere of chromosome 22. The duplicated region includes and extends beyond the CES region, but is only present in three copies. The unbalanced chromosomal constitution results from a 3:1 disjunction event during meiosis I of gamete formation in a parent possessing the balanced translocation [(46,X_, t(11;22)(q23;q11)]. The risk of a translocation carrier producing a child with what is referred to as der(22)t(11q23:22q11) syndrome is approximately 2% (Iselius et al., 1983). Translocation carriers also have an increase in rate of spontaneous abortions. Initially, cases of der(22)t(11;22) syndrome were misclassified as trisomy 22 or "partial trisomy 22" in which the distal portion of the q-arm was assumed to be deleted.

Several large studies in the literature have described the phenotype of the der(22)t(11;22)(q23;q11) syndrome (Iselius et al., 1983; Fraccaro et al., 1980; Zackai & Emanuel, 1980; Schinzel et al., 1981b). Table 3 lists the main features. Phenotypic comparison to other 22q duplication conditions poses a problem because of the additional duplication of 11q23-qter. It is difficult to determine which features are a result of the duplication of proximal 22q and which are the result of duplication of distal 11q. The major similarities to CES will be discussed at the end of this section.

• **Trisomy 22**

Trisomy 22 is the second most common autosomal trisomy found in spontaneous abortuses with a rate of 2.9 per 1000 abortuses examined (Hassold et al., 1980). Like most other autosomal trisomies, it is rarely compatible with life. The majority of trisomy 22 conceptuses likely never reach the point of clinical recognition of a pregnancy (Miller et al., 1980;

Wilcox et al., 1987). Most trisomy 22 conceptuses that do reach the point of recognition of the pregnancy result in spontaneous abortions. Those infants that survive to term have a high postnatal lethality, with the majority of postnatal deaths attributed to "respiratory difficulties".

Numerous early reported cases of trisomy 22 were actually individuals with the der(22)t(11q23;22q11) syndrome, and thus only duplication of proximal 22q. Early cytogenetic analysis did not enable differentiation of the der(22) of this translocation and a normal chromosome 22, and did not allow detection of the der(22) and der(11) in parents carrying the translocation (Bass et al., 1973; Emanuel et al., 1976; Zellweger et al., 1976; Zackai & Emanuel, 1980; Punnett & Kistenmacher 1971 & 1981; Punnett et al., 1973; Alfi et al., 1975). High resolution cytogenetics and in situ hybridization with a chromosome 22 paint are now used to identify trisomy 22s (Slater et al., 1993; Stratton et al., 1993). Prometaphase FISH analysis utilizing subtelomeric probes can also be used to rule out a terminal deletion of 22q (Kobrynski et al., 1993).

Even today, with the molecular means to determine if trisomy for all of chromosome 22 exists, there remains some skepticism as to whether true nonmosaic cases actually survive to birth. To rule out mosaicism many different tissues should be examined, although this is not feasible in many cases. In most cases, both lymphocytes and fibroblasts are examined, and in mosaic individuals, lymphocytes are usually the cells found to be mosaic or disomic (Lund & Traenebjaerg 1990; Pridjian et al., 1995; Lessick et al., 1988; Crowe et al., 1995; Wertelecki et al., 1986). This tissue bias poses difficulties in prenatal diagnosis. Amniocentesis performed because of indications of abnormality on ultrasound results in the examination of cultured cell colonies derived from only a few cells which may not detect mosaicism. Cord blood (lymphocytes) can be drawn, however as described above, may not detect the trisomy and result in an erroneous conclusion of disomy (Désilets et al., 1996). A fetal skin biopsy which is more invasive and dangerous to the fetus may be a better means to detect trisomic cells (Berghella et al., 1996).

In those cases in which an infant non-mosaic for trisomy 22 has survived to term, researchers have stated the importance of testing various regions of the placenta to rule out placental mosaicism or a disomic placenta. Kalousek (1989) suggested that in cases of trisomy 13 and trisomy 18, survival to term is facilitated by a normal placenta or a placenta with some disomic cells which may provide some sort of compensation and enhance the survival of the developing fetus. Phillipson et al (1990) reported two cases of nonmosaic trisomy 22 infants that survived to term, both of which had a mosaic placenta. In contrast, other reports in the literature in which multiple tissues including various regions of the placenta have been examined suggest that a mosaic or disomic placenta is not required for trisomy 22 fetuses to survive to term (Bacino et al., 1995; Slater et al., 1993). Regardless of the reasons why some trisomy 22 individuals survive to term, whether it be due to mosaicism, genetic background or in utero environment, the fact remains that trisomy 22 is generally considered to be a condition incompatible with life because of the high pre- and postnatal lethality.

Table 3. Comparison of the phenotypic features of trisomy 22, the der(22)t(11q23;22q11) syndrome, and CES. Estimates of the frequencies of each feature were calculated by review of the literature.

Malformations/Anomalies		Trisomy 22	der (22) t(11;22)	CES
Development	Mental and psychomotor retardation[1]	severe	severe	mild to moderate
	IUGR (intrauterine growth retardation)	77%	nc	nc
Craniofacial	Ocular coloboma	18%	rare	52%
	Ocular anomalies (microphthalmia, Duane's anomaly)	rare	<10%	36%
	Microcephaly	55%	50%	<10%
	Preauricular skin tags and pits	50%	50%	75%
	Low set/ malformed ears	95%	50%	43%
	Flat broad nasal bridge	59%	nc	14%
	Large nose	nc	33%	nc
	Long philtrum	rare	50%	rare
	Micrognathia (includes retrognathia)	59%	50%	30%
	Palatal anomalies (cleft lip/palate, high arched palate, bifid uvula)	82%	75%	29%
	Hypertelorism	64%	<10%	40%
	Epicanthal folds	59%	<10%	19%
	Downslanting palpebral fissures	27%	<10%	55%
	Short neck and/or redundant skin (webbing)	68%	<10%	<10%
	Hearing loss	nc	rare	14%
Urogenital	Genital anomalies (micropenis, hypoplastic labia, undescended testes, hypospadias, rudimentary intèrnal genitalia)	44% (82% of males)	-	32%
	-micropenis	-	35-90%	-

	-cryptoorchidism	-	30-50%	-
	Renal anomalies (unilateral kidney agenesis, horseshoe kidney kidney hypoplasia, hydronephrosis, ureteral reflux)	68%	20-30%	38%
Cardiovascular	Congenital heart disease (CHD)	77%	40-70%	44%
	Lung hypoplasia	23%	nc	nc
Skeletal	Skull, vertebral, and rib anomalies	27%	20-32%	27%
	Distal limb hypoplasia	32%	nc	nc
	Supernumerary ribs	nc	6-20%	nc
	Hip dislocation	nc	30%	<10%
	Nail hypoplasia	41%	nc	nc
	Fifth finger clinodactyly	23%	nc	<10%
	Long slender fingers	<10%	8-30%	<10%
Gastrointestinal	Anal anomalies (stenosis, atresia, displacement)	27%	13-30%	66%
	Malrotation of the gut	18%	nc	<10%
	Hirschsprung's disease	<10%	nc	<10%
	Diaphragmmatic hernia	<10%	13%	rare
Neuromuscular	Hypotonia	rare	47%	<10%
	Strabismus	rare	26%	12%

nc= not calculated due to no mention of the anomaly in published cases

The lack of information about certain features in clinical reports does not necessarily indicate they are absent.

rare= only a few reports in the literature

[1]Instead of percentages for these features, the degree of affectedness is given as it represents important differences among the duplication conditions.

- **Phenotypic Comparison of Trisomy 22, Der(22)t(11;22)(q23;q11), and CES**

Table 3 summarizes the features observed in all three duplication conditions and demonstrates the phenotypic overlap. Percentages for der(22)t(11q23;22q11) cases were calculated by averaging the values given in Iselius et al (1983), Zackai & Emanuel (1980), Fraccaro et al (1980), and Schinzel et al (1981b) if the values were relatively close. A range is given for those features whose published frequency values were very discrepant. A total of 22 trisomy 22 cases were reviewed (Perez-Castillo et al., 1975; Moro-Serrano et al., 1978; Vohra et al., 1987; Petersen et al., 1987; Voiculescu et al., 1987; Kukolich et al., 1990; M^{c}Pherson & Stetka 1990; Sundareshan et al., 1990; Feret et al., 1991; Kim et al., 1992; Slater et al., 1993; Stratton et al., 1993; Kobrynski et al., 1993; Fahmi et al., 1994; Antle et al., 1990; Nicholl et al., 1994; Isada et al., 1990; Iselius & Faxelius 1978; Bacino et al., 1995; LaDonne et al., 1996). Frequency values for CES were calculated after examination of 77 cases, both published and unpublished. When considering the incidence rates of the features in the table it is essential to bear in mind that these are only estimates based on the characteristics reported in the literature and clinical reports. The absence of a particular feature in a report does not necessarily preclude its absence. As well, some of the features such as hearing loss and strabismus would not be noted in abortuses or cases of early postnatal death and are thus likely underestimates, especially for trisomy 22.

From the table, it is evident that there are both similarities and differences in the phenotypes of these three duplication syndromes. Features of particular interest will be discussed in some detail below.

In terms of development, CES patients are mildly affected relative to the other two conditions, which would be predicted based on the smaller portion of 22q duplicated in CES and the additional distal 11q duplication in the der(22)t(11q23;22q11) syndrome. The majority of trisomy 22 cases have intrauterine growth retardation (IUGR) which is likely the result of a trisomic placenta unable to function in a normal capacity and fulfill the requirements of a developing fetus. Stioui et al (1989) report a

chromosomally normal fetus with a trisomy 22 placenta, born with IUGR but no phenotypic abnormalities.

Many of the features in craniofacial development are present in all three syndromes, however there are some which help distinguish one from another. For example, a short neck with webbed skin is present in the majority of trisomy 22 cases. Epicanthal folds are also more frequently reported in trisomy 22 than in the other two syndromes. A large nose and long philtrum distinguish the der(22) syndrome from CES and trisomy 22. A low incidence of palatal anomalies and a higher incidence of ocular coloboma are characteristic of CES. Of particular interest are the presence of preauricular skin tags and/or pits in all three conditions. Preauricular malformations are considered to be one of the cardinal features of CES due to its presence in the great majority of patients.

Of particular interest is the rarity of ocular coloboma in the der(22) syndrome. In a literature search, only one case in over 150 families was found (Simi et al., 1992), and this case differed from most der(22) cases. The reported individual had one der(11) chromosome and two der(22) chromosomes as a result of second meiotic nondisjunction, in contrast with the one der(22) chromosome in most individuals with this syndrome. It may be that this rare genotype is the reason for the development of the coloboma or that the coloboma is a result of an unrelated chromosomal anomaly or mutation. It is interesting that even though the extent of the chromosome 22 duplications of CES and der(22) individuals are very similar (M°Taggart, this thesis; Barnoski et al., 1995), ocular coloboma is rarely found in the latter. It could be that a duplicated gene on distal 11q influences this. It is unlikely that four copies of the CESCR are required for development of coloboma (the presence of the CEC), since there is a case of CES with an interstitial duplication (three copies of the CESCR) and severe bilateral coloboma (Reiss et al., 1985). Also interesting is the lower incidence of ocular coloboma in trisomy 22 individuals, although this may be the result of the small number of cases reviewed. Several other explanations exist as well. It may be that choroidal or retinal coloboma exists in these individuals but remains undetected or that some compensatory region exists on distal 22q which when duplicated

prevents the development of coloboma. It is also possible that coloboma is found at a much higher rate in the more severely affected individuals which do not survive to a point at which coloboma could be detected.

Incidence of congenital heart defects are slightly higher in trisomy 22 cases than others. Also different are the proportion of types of heart defects in the three conditions. For example, the majority of CES patients with a heart defect have TAPVR or TOF. Der(22)t(11q23;22q11) individuals with a defect most commonly have a septal defect (VSD and ASD) although coarctation of the aorta and pulmonary stenosis are also reported. Trisomy 22 newborns and abortuses have "complex" defects of which ASD, VSD, or TOF are usually a component. Interestingly, TAPVR is rarely reported in either trisomy 22 or the der(22)t(11;22)(q23;q11) syndrome.

Several of the skeletal anomalies differ in frequency among the three conditions. For example, the presence of distal limb hypoplasia, nail hypoplasia, and fifth finger clinodactyly are more specific to trisomy 22, and hip dislocation and long slender fingers are reported more frequently in the der(22) syndrome. Of interest, is the presence of hip dislocation in cases of isolated distal 11q trisomy, suggesting that hip dislocation may be a result of distal 11q trisomy (Pikho et al., 1981). However, Bühler et al (1972) and Kosztolányi & Bühler (1985) report an individual with the supernumerary der(22) of a translocation involving chromosomes 10 and 22 and a CES phenotype with hip dislocation.

One of the cardinal features of CES, anal anomalies, are more frequently reported in CES than trisomy 22 or der(22) syndrome. Cases of inguinal and diaphragmatic hernia are rare in all three conditions as well as Hirschsprung's disease. Hirschsprung's disease is listed here because of the possibility of a susceptibility locus on 22q (Pingault et al., 1997).

◆ Congenital conditions associated with deletions of chromosome 22q

There are also congenital conditions associated with a deletion of a portion of the long arm of chromosome 22. Among these, are a group of syndromes associated with a microdeletion in 22q11.2, just distal to the CES critical region. These include DiGeorge syndrome (DGS) (OMIM#188400), velocardiofacial syndrome (VCFS) (OMIM#192430), conotruncal anomaly face syndrome (CTAFS) (Kinouchi et al., 1976; Burn et al., 1993), and familial and sporadic isolated conotruncal heart defects (Goldmuntz et al., 1993). The deletions associated with these syndromes are believed to be the same. For this reason, Wilson (1993) introduced the acronym "CATCH 22" to encompass DGS, VCFS, CTAFS, and isolated conotruncal heart defects associated with a deletion of a portion of 22q11.2.

C onotruncal heart anomalies
A bnormal facies
T hymic hypoplasia/aplasia
C left palate
H ypocalcemia
22 Deletion of a portion of 22q11.2

A brief summary of phenotypic characteristics associated with each of these conditions is given below, followed by Table 4, demonstrating the phenotypic overlap of these microdeletion syndromes.

- Clinical Features

  - Clinical features of DiGeorge syndrome (DGS)

DGS is a developmental field defect involving derivatives of the third and fourth pharyngeal pouches and arches (Le Douarin 1980; Kirby et al., 1983). Early in embryonic development, a group of multipotential cells called the neural crest cells migrate to the future head and neck region of the embryo. These cells form five "swellings" or arches in the pharyngeal area, thus the term pharyngeal arches (Moore & Persaud, 1993). The

pharyngeal pouches are the grooves on the inside of the arches. The major anomalies of DGS appear to be a result of the abnormal migration of these neural crest cells (Bockman & Kirby 1984). Affected structures include the inferior and superior pairs of parathyroid glands (derived from cells from the third pouch and fourth pouch respectively), the thymus (derived from cells from the third pouch), and the conotruncus, or outflow tract from the heart (derived from the aortic arches of the third and fourth pharyngeal arches) (Moore & Persaud, 1993).

The major anomalies include absence (aplasia) or underdevelopment (hypoplasia) of the parathyroids and/or thymus as well as congenital conotruncal heart defects that typically involve a portion of the outflow tract from the heart (Lammer & Opitz 1986). Parathyroid hypoplasia or aplasia results in hypocalcemia which may manifest as seizures, tremors, or rigidity. Thymus hypoplasia or aplasia results in immune system defects from an increased susceptibility to infections to immunodeficiency. The most commonly reported conotruncal anomalies are interrupted aortic arch (IAA) with ventricular septal defect (VSD) and persistent truncus arteriosus. Also frequently reported are Tetralogy of Fallot (TOF), right aortic arch (RAA), and aberrant right subclavian artery, all of which involve derivatives of the third and fourth pharyngeal arches (Moore & Persaud, 1993).

In addition to these cardinal features of DGS, there are a number of other characteristics occasionally associated with this condition such as short stature, developmental delay, learning difficulties, renal malformations, psychiatric illnesses such as paranoid schizophrenia and depression, hypernasal speech associated with palatal clefting, deafness (both conductive and sensorineural), and a typical facies (OMIM #188400; Wilson D.I. et al., 1993). The most common facial features according to the Oxford Database are telecanthus, low set ears and micrognathia. Also reported are abnormally folded pinnae, short palpebral fissures, short philtrum, bulbous nose with a square nasal tip, and a small mouth in young children (OMIM #188400; Wilson D.I. et al., 1993).

- Clinical features of Velocardiofacial syndrome (VCFS) / Shprintzen syndrome

VCFS is a highly variable congenital condition with a wide range of defects involving all body systems. The most common phenotypic features include palatal clefting, hypernasal speech, learning disabilities, short stature, hypotonia in infancy, a characteristic facies, and congenital heart defects, the most common of which are VSD and conotruncal anomalies (OMIM #192430; Shprintzen et al., 1978; Goldberg et al., 1993). Typical facial features include a long face, a large nose with a bulbous nasal tip, narrow almond-shaped palpebral fissures, external ear anomalies, a small open mouth in young children, a flat malar region and a retrognathia (recessed lower jaw). Other reported features include conductive and sensorineural hearing loss, genital anomalies (hypospadias, undescended testes), inguinal and umbilical hernias, slender hands and digits, scoliosis, and microcephaly (OMIM #192430; Shprintzen et al., 1978; Goldberg et al., 1993).

Ocular anomalies include tortuous retinal vessels, small optic disks, and ocular coloboma. Tortuous retinal vessels do not affect vision and according to Goldberg et al (1993), occur in 30% of VCFS cases. Beemer et al (1986) reported a VCFS individual with ocular coloboma, and there have been a few more reports as well. Cardinal DGS features such as neonatal hypocalcemia, small or absent lymphoid tissue, frequent infections, and T-cell dysfunction have also been reported. Also associated with VCFS is a higher incidence of psychiatric disorders (10% of VCFS Shprintzen et al., 1992) and renal/urological anomalies (DeVriendt et al., 1996). The most common psychiatric disorders include paranoid schizophrenia and depression, the same disorders seen in a higher frequency in DGS patients. Renal anomalies observed are similar to those reported in CES and could be a result of abnormal development of the ureteral bud (DeVriendt et al., 1996).

Many of the minor anomalies found in VCFS are present in the general population and thus if the more serious features of VCFS are not present in an individual, the diagnosis may be missed.

- Clinical features of Conotruncal Anomaly Face syndrome (CTAFS)/ Takao syndrome

The first report of this syndrome was in 1976, when Kinouchi et al. described individuals with characteristic facies associated with conotruncal heart defects. The facial features common to this group of individuals include hypertelorism, narrow palpebral fissures, hypernasal speech and a small mouth. Clinical examination of 50 cases in a report by Burn et al (1993) added some common features including lateral displacement of the inner canthi, bloated eyelids, low nasal bridge, high arched palate, malformed ears, prominent ears, and mild mental retardation.

Table 4. Features of the CATCH 22 microdeletion syndromes. A (+) or a (-) sign denotes the presence or absence of a characteristic. The more common, or cardinal features of each syndrome are in shaded boxes with the exception of facial features which are marked present or absent for comparison of the four conditions.

	DGS	VCFS	CTAFS	Isolated CHD
Mental retardation	+	+	+	-
Learning difficulties	+	+	-	-
Developmental delay	+	+	-	-
Short stature	+	+	-	-
Hearing loss	+	+	-	-
Psychiatric illness	+	+	-	-
Hypocalcemia	+	+	+	-
T-cell deficiency/	+	+	+	-
frequent infections	+	+	+	-
Cleft palate	+	+	+	-
Hypernasal speech	+	+	+	-
Conotruncal CHD	+	+	+	+
VSD	+	+	+	+
Renal anomalies	+	+	-	-
Genital anomalies	+	+	-	-
Slender digits	-	+	-	-
FACIAL FEATURES				
Microcephaly	-	+	-	-
Long face	-	+	-	-
Flat malar region	-	+	-	-
Micrognathia	+	-	-	-
Retrognathia	-	+	-	-
Tortuous retinal vessels	-	+	-	-
Bloated eyelids	-	-	+	-
Short palpebral fissures	+	-	-	-
Narrow palp. fissures	-	+	+	-
Ear anomalies/	+	+	+	-
low set	+	?	-	-
malformed	+	?	+	-
prominent	-	?	+	-
Bulbous nose	+	+	-	-
Square nasal tip	+	-	-	-
Low nasal bridge	-	-	+	-
Prominent nose	-	+	-	-
Short philtrum	+	-	-	-
Small mouth	+	+	+	-

• Etiology

• Etiology of DGS and VCFS

DGS is causally heterogeneous, associated with several chromosomal anomalies as well as in utero exposure to teratogens and certain conditions. For example, maternal diabetes, retinoic acid and alcohol can all lead to the birth of infants with DGS-like features (Lammer et al., 1985; Binder 1985; Wilson T.A. et al., 1993; Gosseye et al., 1982; Novak & Robinson 1994). Chromosomal anomalies other than del(22)(q11.2) associated with the DGS phenotype are deletions for a 2Mb region of 10p13 (Greenberg et al., 1988a; Lai et al., 1992; Daw et al., 1996), a portion of 17p, and part of the q-arm of chromosome 4 (Greenberg et al., 1988b; Monaco et al., 1991; Fukushima et al., 1992).

These causes of DGS account for a very small proportion of cases. DGS is most commonly associated with a deletion of part of 22q11.2. Deletions can result from both visible and microdeletions as well as unbalanced translocations. The first association of 22q11.2 with DGS involved patients with unbalanced translocations and visible interstitial deletions (de la Chapelle et al., 1981; Kelley et al., 1982; Greenberg et al., 1988b).

Only an estimated 15% to 20% of the deletions are visible using high resolution cytogenetics (Desmaze et al., 1993; Greenberg et al., 1988b; Wilson et al., 1992a). Molecular analysis using quantitative dosage (Driscoll et al., 1992a), FISH (Desmaze et al., 1993) and hemizygosity studies (Scambler et al., 1991) has enabled detection of the microdeletions. Deletions in 22q11.2 are estimated to account for approximately 90% of DGS patients (Driscoll et al., 1992a; Desmaze et al., 1993; Driscoll et al., 1993).

Like DGS, VCFS is also likely causally heterogeneous, although the only chromosomal anomaly found associated with VCFS so far is the 22q11.2 deletion. Approximately the same proportion of VCFS as DGS have visible interstitial deletions of this region (Driscoll et al., 1992b)

Microdeletions of the region are detectable by RFLP, quantitative dosage and FISH analyses. The percentage of individuals with VCFS and a 22q11.2 deletion was estimated to be 76% (Driscoll et al., 1993), suggesting that a small proportion of individuals with a VCFS-like phenotype have another cause.

Presented below in Figure 4 is a summary of the findings in deletion studies of VCFS and DGS. The majority of deletions can be placed in one of two categories based on the extent of the deletion. A concept reiterated in every recent article concerning deletion characterization in DGS and VCFS is that there are no phenotypic differences between individuals of the two groups; the larger deletions are not more severely affected than smaller deletions. (Driscoll et al., 1992a, 1992b; Scambler et al., 1991; Carlson et al., 1997).

The most common deletion is the larger one which is approximately 2 to 3Mb in size, flanked by loci D22S427 and D22S306/308 (Morrow et al., 1995; Carlson et al., 1997) or D22S427 and D22S636 (Driscoll et al., 1995). Refer to Figure 4 for a depiction of this region and the deletions. A 1.5 to 2Mb deletion is the less common smaller deletion, encompassing the low copy repeats designated sc11.1a and sc11.1b, and flanked by loci D22S427 and D22S264 (Lindsay et al., 1993; Halford et al., 1993b; Morrow et al., 1995; Halford et al., 1993a; Driscoll et al., 1993) or D22S427 and BCRL-2 (Driscoll et al., 1995).

The actual breakpoints themselves appear to cluster within an interval. In both size groups of deletions, there appears to be one proximal breakpoint interval and two possible distal intervals. Determination of the precise location of each interval is made somewhat difficult due to different research groups performing the analyses with different probes. Both research groups found the same proximal deletion breakpoint interval for the larger and smaller deletions. The interval is flanked by locus D22S427 and either D22S36 (Driscoll et al., 1995), D22S1638 (Carlson et al., 1997), or D22S941 (Morrow et al., 1995). The distal breakpoint interval of the smaller deletions is flanked by D22S944 and D22S264 (Morrow et al., 1995) or D22S259 and BCRL-2 (Driscoll et al., 1995). The

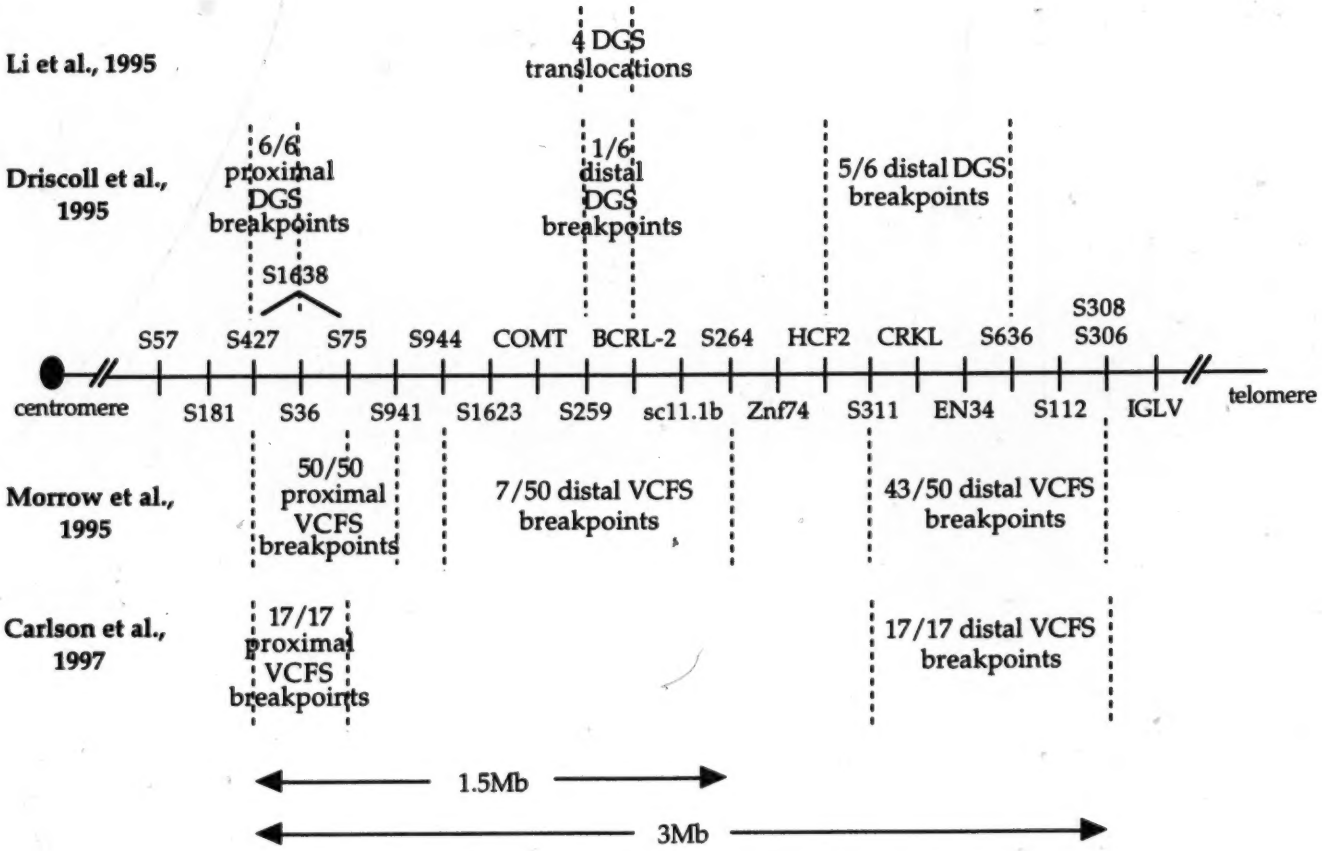
breakpoints of four DGS translocations have been localized to the D22S259 to BCRL-2 interval as well (Li et al., 1995). The loci flanking the distal breakpoint intervals of the larger deletions were defined by Morrow et al (1995) and Carlson et al (1997) as D22S311 and D22S306/308, and by Driscoll et al (1995) as HCF2 and D22S636. Kurahashi et al (1996) performed deletion analysis of 37 individuals and found that 33 of these individuals had a larger deletion and 3 had a smaller one (the other patient had a translocation). Although the probes used in the analysis were not as useful for the specific localization of the deletion breakpoints, the analysis did show that the two sizes of deletions had similar proximal breakpoints, with the size difference of the deletion being attributed to the location of the distal breakpoint.

By comparing deletions, both interstitial and those resulting from unbalanced translocations, a smallest region of deletion overlap has been defined (SRO). This SRO differs from report to report and does not always represent the full spectrum of either DGS or VCFS. This region did however, serve as the starting region in the search for genes. Table 5 lists some of the genes isolated which may play a critical role in the development of DGS/VCFS.

- Etiology of Conotruncal Anomaly Face syndrome

In 1993, Burn et al., found an association between individuals with CTAFS and a deletion in 22q11.2. These microdeletions were not visible by high resolution cytogenetics but were detected by FISH analysis. Deletions were detected using probes such as D22S75 (N25) and D22S259 (R32), known to be deleted in virtually all cases of DGS and VCFS (Driscoll et al., 1992a; Driscoll et al., 1992b). Matsuoka et al (1994) found D22S75 deletions by FISH analysis in 42 of 50 CTAFS patients tested. Seaver et al (1994) found a deletion of D22S264 in four patients tested.

Figure 4. Summary of DGS/VCFS deletion analyses. The relative order of loci on this map has been constructed from Collins et al (1995), Budarf et al (1996), and Carlson et al (1997). Loci beginning with an "S" should be preceeded by D22. The exact location of D22S1638 is unknown relative to D22S36 but is flanked by D22S427 and D22S75. The map is not drawn to scale. Above the map are breakpoint intervals obtained by Driscoll et al (1995) in the examination of six families with DGS and the locations of four DGS translocations (Li et al., 1995). Below the map are VCFS deletion breakpoint intervals found in two studies carried out by the same group of individuals. In an examination of 61 VCFS patients by Morrow et al (1995), 50 were found to have deletions in 22q11.2. Carlson et al (1997) characterized 17 interstitial deletions in VCFS patients with psychiatric disorders. Two deletion sizes exist: the smaller deletion spans approximately 1.5Mb whereas the more common larger deletion spans approximately 3Mb.


Li et al., 1995
4 DGS translocations
Driscoll et al., 1995
6/6 proximal DGS breakpoints
1/6 distal DGS breakpoints
5/6 distal DGS breakpoints
S1638
S57
S427
S75
S944
COMT
BCRL-2
S264
HCF2
CRKL
S636
S308
S306
centromere
S181
S36
S941
S1623
S259
sc11.1b
Znf74
S311
EN34
S112
IGLV
telomere
Morrow et al., 1995
50/50 proximal VCFS breakpoints
7/50 distal VCFS breakpoints
43/50 distal VCFS breakpoints
Carlson et al., 1997
17/17 proximal VCFS breakpoints
17/17 distal VCFS breakpoints
1.5Mb
3Mb

- Etiology of isolated conotruncal congenital heart defects (sporadic and familial)

Due to the common feature of conotruncal congenital heart anomalies in DGS, VCFS, and CTAFS, and the common etiology, it was suggested that an important gene affecting the development of the outflow tract of the heart was situated in 22q11.2. If this were indeed the case, it was hypothesized that some cases of isolated conotruncal heart defects of the type seen in DGS/VCFS also possessed microdeletions in this region. In a study performed by Goldmuntz et al (1993), patients with non-familial isolated interrupted aortic arch (IAA) or tetralogy of Fallot (TOF) were selected and tested for deletions using probes in the 22q11.2 area deleted in DGS and VCFS patients (N25, pH160b, and R32, in a proximal-distal order). Five of 17 individuals were in fact deleted for the probes tested, thus confirming the hypothesis that the 22q11.2 region was important to the development of the outflow tract of the heart. In a study of familial cases of isolated outflow tract defects, Wilson et al (1992b), found that five of nine families had 22q11 deletions.

Despite the overlap of features and the possible common etiology, the above microdeletion syndromes are still considered to be clinically distinct. Hall (1993) argues that these conditions are all the same because of the common etiology and that a bias of ascertainment results in classification as different syndromes. It is plausible with what we know about other syndromes, that deletion of the same genes could produce the variability observed in these syndromes as a result of environmental influences, genetic background, and stochastic factors.

Table 5. Summary of genes and their putative functions which may play a role in the development of the DiGeorge and Velocardiofacial syndromes.

Gene Name(s)	Putative Function	Reference(s)
CTP	Mitochondrial citrate transporter protein	Heisterkamp et al., 1995
DGCR6	Homology to human laminin: tissue assembly, cell migration, attachment and differentiation	Demczuk et al., 1996
IDD/LAN	Transmembrane protein	Budarf et al., 1995 Wadey et al., 1995 Demczuk et al., 1995
HIRA/TUPLE	Regulation of transcription	Halford et al., 1993 Lamour et al., 1995
ES3/CLTCL	Clathrin heavy chain-like gene	Lindsay et al., 1996
DVL-22	Homology to Drosophila polarity *dsh* gene	Pizzuti et al., 1996
DGCR5	Several splice variants but no protein product: may be a functional RNA product: -a transcriptional regulator of more distal genes or a nucleation center	Sutherland et al., 1996
GSCL	*Goosecoid* -like homeobox gene (transcription factor)	Gottlieb et al., 1997

• Research Objectives

The purpose of my research was to localize the breakpoints in 22q11.2 of the duplications associated with cat eye syndrome. My work focused mainly on the most common form of the duplication, the cat eye chromosomes.

• type I CECs

Can the two breakpoints of each CEC be further localized than in Mears et al (1994), using a new locus in the 1Mb interval?

• type II CECs

How far do these duplications extend into the CATCH22 syndrome deletion region, and are they occurring in the same region?

Are there any obvious phenotypic differences from individuals with the type I CECs?

How closely do these CEC duplication breakpoints correspond to the CATCH22 syndrome deletion breakpoints?

Is there evidence of rearrangement "hotspots" in a region known to contain low copy repetitive sequence elements?

MATERIALS AND METHODS

Human Cell/Tissue Culture:

The patients studied and their parents are listed in Tables 6 and 7 respectively. Cell lines or cultures were available for most of these individuals.

All cultures were incubated in a 37°C humidified incubator (Forma Scientific Water-Jacketed Incubator), supplied with 5% carbon dioxide (CO_2). Epstein-Barr virus (EBV) transformed lymphoblastoid cell lines were cultured in RPMI 1640 supplemented with 1% L-glutamine, 1% penicillin/streptomycin, and 10% to 20% fetal bovine serum (FBS). Monolayer cultures were grown in DMEM (Dulbecco's modified Eagle medium) or MEM (minimal essential medium) supplemented with 1% L-glutamine, 1% penicillin/ streptomycin, and 10% to 20% FBS (all reagents from GIBCO-BRL).

Freezing Cells

Lymphoblasts and fibroblasts were frozen in essentially the same manner. One T25 flask of lymphoblastoid cells was frozen in one cryovial and one T75 flask of fibroblasts was divided into two cryovials. Flasks were checked very carefully for contamination before freezing and only flasks containing cells appearing healthy were chosen. For lymphoblastoids, flasks with yellow media were not used as this was an indication that the cellular environment was acidic, and the cells needed to be fed. Fibroblasts were frozen when they reached 70% confluency (K. Romanyk, personal communication).

The media was removed from the cells by centrifugation. The cells were completely resuspended in 1 ml of 6% DMSO (dimethyl sulfoxide - C_2H_6OS SIGMA#D-2650) in FBS or in media containing 20% FBS (K. Romanyk, personal communication). The cryovials were frozen slowly in a styrofoam box containing liquid nitrogen where they remained

suspended in the vapor for 0.5 to 3 hours before being transferred into liquid nitrogen storage (Rooney & Czepulkowski, 1986). Normally, one vial was thawed and cultured to ensure the cells were healthy and free of contamination.

Thawing cells

After removal from liquid nitrogen storage, vials were quickly thawed in a 37°C water bath to avoid the formation of ice crystals (Rooney & Czepulkowski, 1986). The cells were then transferred to a flask containing media. After incubation for a period of time that allowed the lymphoblastoid cells to settle in the flask or the fibroblasts to adhere to the flask, the media containing DMSO was removed and fresh media added.

EBV Transformation

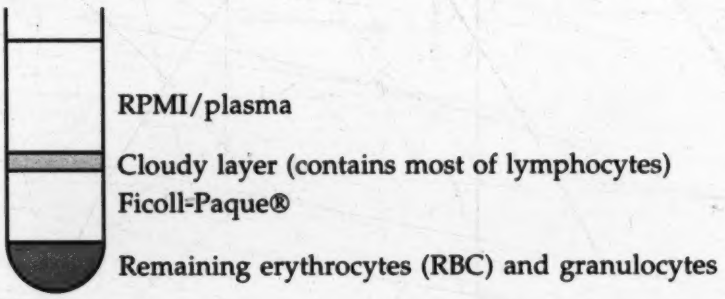
Epstein-Barr virus (EBV) was used to immortalize or transform lymphocytes, establishing a lymphoblastoid cell line. EBV selectively transforms B-lymphocytes (Epstein et al., 1964; Pattengale et al., 1973; Sixbey et al., 1983).

The procedure for lymphocyte isolation was compiled by H. M'Dermid from the Ficoll-Paque® instruction manual, and from personal communication with numerous individuals. Peripheral blood samples (5 ml) were spun for 5 minutes at 2500 RPM to separate three layers (from top to bottom of tube): plasma (no cells), the " buffy" coat (leukocytes), and red blood cells (erythrocytes) plus granulocytes. The bottom portion of the plasma, the buffy coat, and the top portion of the erythrocyte / granulocyte layer were transferred to a new tube, brought up to a volume of 4 ml with RPMI 1640 medium supplemented with 1% penicillin / streptomycin, and layered over 4 ml of Ficoll-Paque® (Ficoll-sodium diatrizoate solution, Pharmacia). The solution was spun for 20 minutes gradually increasing the speed to 2000 RPM over the first few minutes. Figure 5 depicts the layers evident after centrifugation.

Most of the plasma/RPMI layer was discarded. The remaining plasma/RPMI, as well as the cloudy layer containing the lymphocytes was transferred to a fresh tube and brought up to 10 ml with RPMI 1640 supplemented with 1% penicillin/streptomycin. This mixture was centrifuged at 800 RPM for 8 minutes and then the RPMI was removed. This "wash" was done twice more and then the cells were resuspended in 5 ml RPMI + 20% FBS + 1% penicillin/streptomycin + 1% L-glutamine and incubated in a T25 flask at 37°C for 4 to 8 hours to recover.

After recovery, approximately 0.01 mg of cyclosporin (F. Bamforth, University of Alberta Hospital) and approximately 6×10^5 particles of EBV (Tampa Bay Research Institute) were added. The flask was left undisturbed for several days. Media color (pH) was monitored over the following several weeks. When a change was noticed, cells were checked for growth (the presence of doublets or clumps, healthy cells with the characteristic morphology of transformed cells). Cells were fed until ready to split and then split 1:3 or 1:5 depending on growth.

Figure 5. Lymphocyte isolation. The layers present after centrifugation of the initial "buffy" layer extracted from a peripheral blood sample, layered over a Ficoll-Paque® solution.



Establishment of Fibroblast Culture

This procedure is a combination and modification of those described in Current Protocols in Human Genetics Volume 2 Section 8·7·6 and Rooney & Czepulkowski (1986).

A skin biopsy was obtained from the abdomen of patient CM20 during a surgical procedure to correct a colostomy. The sample was stored in media overnight before setting up the culture.

The tissue was placed in a plastic petri dish in a drop or two of media to prevent drying, and then was cut into small pieces (1 mm x 1 mm) using two scalpels. Half of the tissue pieces were briefly dipped into Fungizone® solution (GIBCO/BRL) as suggested by L. Enns (personal communication) and then transferred to a dish containing medium.

The undersides of fresh plates were scored with a scalpel to mark the locations of the tissue pieces (Figure 6).

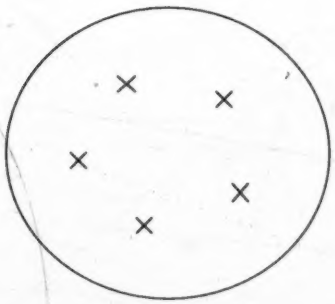


Figure 6. Depiction of a tissue culture plate with the locations of tissue pieces found to give best growth

Each tissue piece was positioned on a marked spot in a very small drop of media and the plate placed at 37°C until the tissue had adhered (the media surrounding the tissue had almost completely evaporated). Enough media (DMEM supplemented with 20% FBS, 1% L-glutamine, 1%

penicillin/streptomycin) to cover the tissue was carefully added and the next day, the tissue pieces were checked for adherence. After a few days, the cultures were checked under the microscope for growth. If the tissue was healthy and the cells were continuing to divide, there was a dense aggregation of cells (likely not fibroblasts) surrounding the piece of tissue. Some fibroblasts extended beyond this dense cell aggregation. During growth of the culture the media collected considerable cellular debris, thus when the cells were fed, the media was replaced. The culture took several weeks to begin growing at a faster rate. Upon confluency of the plate (assuming the tissue pieces were set up as above and all the pieces had adhered and were growing), the cells were trypsinized without disturbing the tissue (it was quite difficult to remove the tissue with normal trypsinization procedures). The trypsin was neutralized with two volumes of media and transferred to either a T75 or T25 flask, depending on the quantity of cells. The media was replaced in the original plate, as the tissue continued to produce fibroblasts. The cells obtained from the original plate were termed "first passage". When the flasks were confluent, they were split 1:3. Cells were frozen as soon as possible in case contamination developed and because fibroblasts have a limited number of passages before senescence.

Table 6. Listing of all individuals studied in this project. The original source is the referring physician or investigator. The cell line source is the investigator from whom the transformed lymphoblastoid cell line or established monolayer culture was received. Both Tables 1 and 2 are formatted after Table 5 in Mears (1995).
The individuals within the shaded areas are new additions since Mears (1995).

Individuals used as control samples

Lab ID	Code	Cell type	Original source	Cell line source
	GM03657	Lymph	L. Coriell	NIGMS[1]
	GM02325	Fibroblast	A. Al Saadi	NIGMS
	GM07106	CVS[2]	P. Jacobs	NIGMS

CES individuals with a type I supernumerary CEC (cat eye chromosome)

Lab ID	Code	Cell type	Original source	Cell line source
MM	CM04	Lymph	Dr. R. Stallard	Dr. A.M.V. Duncan
S2	CM05	Lymph	Dr. A. Schinzel	Dr. A.M.V. Duncan
S5	CM06	Lymph	Dr. A. Schinzel	Dr. A.M.V. Duncan
GM	CM07	Lymph	Dr. J. Siegel-Bartelt	Dr. J. Siegel-Bartelt
JM	CM08	Lymph	Dr. J. Siegel-Bartelt	Dr. J. Siegel-Bartelt
IG	CM09	Lymph	Dr. M. Baraitser	Dr. A.M.V. Duncan
ISCA	CM10	Lymph	Dr. V. Van Heyningen	Dr. V. Van Heyningen
MG	CM11	Lymph	Dr. B.S. Emanuel	Dr. B.S. Emanuel
JOC	CM17	Blood	Dr. E. Quackenbush	n/a

CES individuals with a type II supernumerary CEC

Lab ID	Code	Cell type	Original source	Cell line source
BZ	CM01	Lymph	Dr. R.S. Verma	Dr. A.M.V. Duncan
JD	CM02	Lymph	Dr. C.R. Greenberg	Dr. H.E. McDermid
KS	CM03	Lymph	Dr. W.J. Rhead	Dr. J. Biegel
BR	CM20	Fibroblast	Dr. P. Ferreira	K.E. McTaggart

CES individuals with a supernumerary ring chromosome

Lab ID	Code	Cell type	Original source	Cell line source
25105	CM15	Lymph	Dr. S.R. Patil	Dr. S.R. Patil
ME	CM16	Lymph	Dr. Y. Fukushima	Dr. Y. Fukushima

[1] NIGMS= Human Genetic Mutant Cell Repository, Coriell Institute for Medical Research
[2] CVS= chorionic villus sample
n/a= not available

Table 7. Parents of CES patients. The denotation "Y" as the first letter of the code indicates father and "X" indicates mother. For example, individual XM20 is the mother of patient CM20.

Lab ID	Code	Cell type	Original source	Cell line source
ROC	YM17	Blood	Dr. E. Quackenbush	n/a
TAC	XM17	Blood	Dr. E. Quackenbush	n/a
MoF	YM20	Lymph	Dr. P. Ferreira	K.E. M°Taggart
MR	XM20	Lymph	Dr. P. Ferreira	K.E. M°Taggart

Human Genomic DNA Preparation

From blood + heparin or EDTA

This was a large scale version of the procedure described by Gustincich et al (1991). The scaling up of this procedure was done by Mears (1995).

To a 5 ml peripheral blood sample, 10 ml of blood lysis buffer (Appendix A) was added and mixed by inversion. Samples were placed at 68°C for 10 minutes. Immediately, 15 ml of chloroform was added, mixed by inversion, and centrifuged 15 minutes at 10,000 RPM in an HB-4 swinging bucket rotor. The aqueous upper layer was transferred to a fresh tube with a pipette. To this aqueous layer, 15 ml of sterile water and 1.7 ml of a 5% CTAB solution (Appendix A) were added, mixed by inversion and centrifuged 10 minutes at 10,000 RPM. The supernatant was decanted and 5 ml of a 1.2 M sodium chloride (NaCl) solution was added to the pellet which was allowed to resuspend at room temperature or 37°C overnight on a nutator. Cold 95% ethanol (EtOH) (18 ml) was added, mixed by inversion, and the DNA was spooled out. The DNA was washed with 70% EtOH, dried under vacuum, and the pellet resuspended in 300 ul to 500 ul TE (1 mM ethylenediaminetetra-acetic acid disodium salt (EDTA)/ 10 mM Tris-HCl, pH 8.0). Spectrophotometer readings (A_{260} and A_{260}/A_{280}) were taken and a digested and non-digested sample subjected to agarose gel electrophoresis to check for the ability of restriction enzymes to cleave the DNA, shearing, presence of RNA, and to confirm concentration.

From cultured fibroblasts or lymphoblastoid cell lines

This preparation was the same as that above except after harvest of the cells (amalgamation of flask contents, centrifugation, removal of media, and washing once with Hank's Balanced Salt Solution/HBSS, GIBCO), 5 ml of HBSS (Appendix A) was added to resuspend the cells followed by 10 ml of the blood lysis buffer. Incubation at 68°C was for 25 minutes and two chloroform extractions were usually required. For

resuspension of the precipitated DNA pellet, 300 ul to 800 ul of TE was added. Normally, 3 to 5 saturated T25 flasks were harvested when using lymphoblastoid cells yielding an average of 500ug of DNA, and 7 to 10 confluent T75 flasks when preparing DNA from fibroblasts yielding 200ug to 500ug of DNA.

Cosmid DNA Preparation

Two methods were used to prepare cosmid DNA

Small Scale Alkaline Lysis

This procedure is a modification of that in Sambrook et al (1989).

A 5 ml solution of Luria-Bertani (LB) medium (Appendix A) (+ 10 ug/ml kanamycin) was inoculated with a single isolate from a bacterial petri plate containing the same medium and grown overnight or all day at 37°C. The cells were pelleted by centrifugation and the supernatant discarded. The cell pellet was resuspended in 150ul TGE (Appendix A) by vortexing, followed by the addition of 300 ul of a 200 mM sodium hydroxide (NaOH)/ 1% SDS (sodium dodecyl sulfate) solution and mixing by inversion. Addition of 250 ul of a 3 M potassium acetate (KOAc) solution and mixing by inversion was followed by placement of the solution on ice for 10 minutes. Centrifugation for one minute was followed by treatment of the supernatant with 60 ug of RNase A at 37°C for 30 to 45 minutes, followed by phenol, and then chloroform extractions, or by phenol, phenol/chloroform, and then a chloroform extraction. The aqueous portion was precipitated by the addition of two volumes of 95% EtOH and placement at -70°C for 30 minutes. After centrifugation, a 70% EtOH wash was performed, followed by centrifugation and drying of the DNA pellet under vacuum. The DNA pellet was resuspended in 50 to 100 ul of TE. Spectrophotometer readings were carried out (A_{260}, $A_{260/A280}$), and restricted and non-restricted aliquots tested by agarose gel electrophoresis.

Large Scale QIAGEN

The large-scale preparation described in the Qiagen manual was used, starting with a 500 ml culture.

Both cosmid DNA procedures gave DNA of the same quality. In terms of quantity, the Qiagen preparation resulted in an average yield of 90 ug of DNA per 500 ml of culture and the alkaline lysis miniprep gave an average yield of 62 ug per 5 ml of culture. The Qiagen preparation was thus significantly less efficient considering the initial quantity of saturated culture from which the cosmid DNA was extracted.

YAC-Containing Yeast Genomic DNA Preparation

Saccharomyces cerevisiae strains containing human YACs and mega YACs were grown in 5 ml of SD medium (Appendix A) at 30°C overnight from a single isolate. The majority (4 ml) of this culture was used to inoculate 60 ml of fresh medium, and the rest (1 ml) was used to prepare a frozen glycerol stock. This culture was grown for 36 to 48 hours at 30°C and used to prepare both DNA agarose plugs for pulsed field gel electrophoresis (PFGE) analysis and for DNA to be used in fluorescent *in situ* hybridization (FISH) analysis (most DNA and agarose plug preparations were performed by D. Shkolny and A. Johnson). It was necessary to perform both PFGE and FISH experiments with DNA prepared from the same culture as several of the YACs were found to be unstable, yielding YACs of different sizes in different isolates.

DNA Preparation for FISH

This procedure is a modification of Section IV 13.11.1 in Current Protocols in Molecular Biology, Volume 2. Two thirds of the culture was centrifuged at 5,000 RPM for 5 minutes to pellet the yeast cells (one-third of the culture was used for the preparation of DNA plugs for PFGE). The supernatant was discarded and the cell pellet resuspended in 3 ml of a 0.9 M sorbitol/ 0.1 M EDTA (pH 7.5) solution. Lyticase (0.3 mg) was added and incubated for one hour at 37°C. The cells were repelleted and resuspended

in 5 ml of a 50 mM Tris-Cl (pH 7.4)/ 20 mM EDTA solution. 0.5 ml of 10% SDS was added and incubated at 65°C for 30 minutes to lyse the cells. Following this incubation, 1.5 ml of a 5 M potassium acetate (KOAc) solution was added and the solution placed on ice for one hour and then centrifuged 10 minutes at 10,000 RPM. The supernatant was filtered into a fresh tube, two volumes of room temperature 95% ethanol added, and centrifuged 15 minutes at room temperature at 5,000 to 6,000 RPM. The supernatant was discarded, and the nucleic acid pellet was dried and resuspended overnight in 3 ml of TE. The next day, the solution was centrifuged 15 minutes at 10,000 RPM, then the supernatant transferred to a fresh tube and treated with 0.15 mg of RNase A at 37°C for 30 minutes. The DNA was precipitated with one volume of isopropanol, removed from the tube, air dried in an eppendorf tube, and then resuspended in 0.5 ml of TE. The DNA was then fluorescently labeled for FISH analysis (described in materials and methods FISH section).

Restriction Enzyme Digestion

Restriction endonucleases from BRL, New England Biolabs, and Pharmacia were used under the suggested conditions with the appropriate buffers. Bovine serum albumin (BSA) and spermidine at final concentrations of 100 ug/ml and 4 mM, respectively, were used in all restriction endonuclease digests.

Southern Transfer

Two methods of transfer were used. The first was that described by Southern (1975), involving denaturation (0.5 M NaOH/1.5 M NaCl) and neutralization (3 M NaCl/ 0.5 M Tris-HCl, pH 7.5) of the agarose gel followed by transfer of the DNA fragments to a nylon membrane (Genescreen™ Plus membrane, DuPont) in a salt solution (10X SSC). The second method consisted of alkaline (0.4 M NaOH) treatment of the gel followed by transfer in the same alkaline solution (Genescreen Plus™ manual & Current Protocols in Molecular Biology. Volume I, Section 2.9.7)

The first method was used for most normal agarose gels and all of the PFGE agarose gels. To assist in the transfer of the large DNA fragments in PFGE, the agarose gel was first depurinated in a 0.25 M hydrochloric acid (HCl) solution for 15 minutes which resulted in "nicking" of the DNA (McDermid et al., 1993). The alkaline method (second method) had the advantage of requiring less time than traditional Southern blotting and no differences were observed in the efficiency of transfer, although stringent experiments comparing the two methods were not carried out.

After overnight transfer, the membrane was treated with a 0.4 M NaOH solution for 1 minute followed by a 0.2 M Tris, 0.1X SSC, 0.1% SDS solution for 5 minutes and then placed between two pieces of 3MM Whatmann until dry (Genescreen Plus™ Manual).

Southern Hybridization

Fragments for use as probes were generated by restriction endonuclease digestion of DNA (plasmid, phage, cosmid) and electrophoresis in low melt agarose (FMC 50102). The desired fragment was excised from the gel with a scalpel and stored at -20°C (Feinberg & Vogelstein, 1984).

Radioactive labeling was achieved using a modification of the random priming method described by Feinberg & Vogelstein (1983 & 1984), which utilized hexamer primers and the large fragment of DNA polymerase I. Isolated probe DNA in low melt agarose was denatured by boiling for 10 minutes. While still liquefied, 20 to 30 ul of this probe was combined with 10 ul OLB (oligo-labeling-buffer, Appendix A), 2 ul 10X BSA, 5 ul of α-^{32}P-dCTP, 11.8 units of Klenow and incubated at 37°C for one to two hours (M. Higgins, personal communication and, a modification of Feinberg & Vogelstein 1984). After this period, a stop solution (20% glycerol/ 67 mM EDTA/ blue dextran) was added to inhibit the enzyme, and the probe solution boiled for 2 minutes to ensure that the agarose was completely liquefied. To remove unincorporated α-^{32}P-dCTP, the reaction was passed over a column made of 1.5 ml microfuge tubes

depicted in Figure 7, containing Sephadex G-50 (Pharmacia 17-0045-02) in TE (modification of Feinberg & Vogelstein 1983 and M. Higgins, personal communication). The columns were spun at 800 RPM for 2 minutes, after which the incorporation value was roughly estimated by comparing Geiger counter readings of the top and bottom portions of the column. If necessary, repetitive sequences were competed out by preannealing the probe with 1.25 mg (500 ul of a 100 mg/ 40 ml solution) of nonradioactive sheared/ sonicated placental DNA (Litt & White 1985). The probe/ placental DNA solution was boiled for 10 minutes to denature the DNA and allowed to preanneal at 65°C for 1 to 6 hours.

Membranes were prepared for hybridization by moistening with stripping wash (0.2 M Tris/ 0.1X SSC/ 0.1% SDS), followed by placement in a hybridization bottle with 6 to 10 ml of a modification of Church & Gilbert's prehybridization solution described in M^{c}Dermid et al., 1993 (Appendix A), and then incubated at 65°C for 1 to 4 hours.

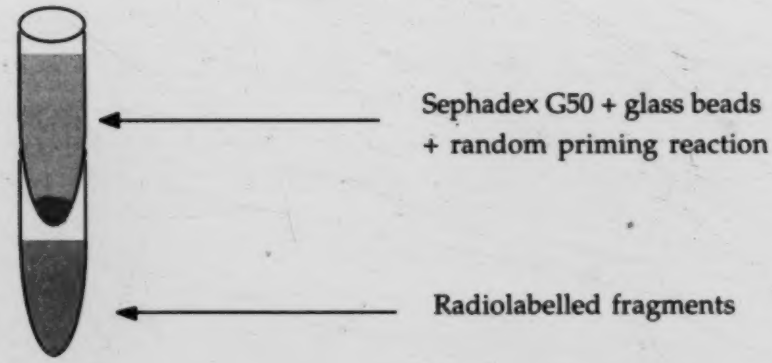
If the probe was preannealed it was added directly to the blot. If not, it was boiled for 10 minutes to denature the DNA, and then added to the prehybridized membrane and incubated at 65°C overnight (16 to 20 hours).

Following hybridization the membrane was washed twice at room temperature for 10 minutes in a 1.5X SSC/ 0.2% SDS solution. Two additional 10 minute washes at 65°C were carried out in a 0.2X SSC/ 0.2% SDS solution. The blots were checked with the Geiger counter and if necessary, another wash at 65°C may have been performed with a 0.1X SSC/ 0.2% SDS solution (H. M^{c}Dermid, personal communication; a modification of Current Protocols in Molecular Biology Volume 1 Section 2.9.6, and Sambrook et al., 1989). Following the washes, the blots were sealed in a plastic bag and placed in an autoradiography cassette with Kodak X-OMAT AR film at -70°C for an appropriate length of time and then developed using a Kodak M35A X-OMAT processor.

Most membranes were probed more than once. Radioactively labeled DNA could be removed from the membrane by one of two methods. The least stringent of these involved incubation at 42°C to 45°C

for 30 to 40 minutes in a solution of 0.4 M NaOH and then 30 to 40 minutes in a 0.2 M Tris/ 0.1X SSC/ 0.1% SDS solution (Genescreen Plus™ manual). The more stringent of the two methods entailed boiling the membrane for 30 minutes in a 1% SDS/ 0.1X SSC solution. Regardless of the method used, membranes were checked before and after the stripping procedure with a Geiger counter to confirm removal of the radioactively labeled DNA.

Figure 7. Depiction of the spin column used to eliminate unincorporated nucleotides from random priming radioactive labeling reactions. The top microfuge tube has a hole punctured in the bottom to enable passage of radiolabelled probe through the Sephadex into the empty bottom tube. The top tube contains a small quantity of glass beads in the bottom to prevent the Sephadex from passing through as well.



Quantitative Dosage Analysis

Quantitative dosage analysis was performed as described previously in Mears et al (1994). Briefly, four to eight restriction enzyme digestion replicates were set up for 2-copy control (GM03657), 3-copy control (GM02325 or GM07106), and CES individuals. After agarose gel electrophoresis and Southern transfer to Genescreen Plus™ membrane, hybridization was performed with radioactively labeled test and control probes (see Table 8) overnight. Following washes to remove excess probe and exposure to autoradiographic film for an appropriate length of time, densitometric analysis was performed using a BIORAD Model GS-670 Imaging Densitometer and Molecular Analyst software version 1.4. The average signal across each lane of the autoradiograph was determined and the ratios of test to control in each lane were calculated and pooled for each individual. Comparisons to the other individuals were done using the Wilcoxon rank sum test (Wilcoxon 1945; Wilcoxon & Wilcox 1964), a non-parametric statistical analysis which ranks the ratios for two individuals and determines whether the two data sets are different.

Fluorescence in situ hybridization (FISH)

Probes used for FISH were derived from several sources: phage, cosmids and total yeast genomic DNA containing yeast artificial chromosomes (YACs). Table 9 lists the cosmid addresses and phage used as probes, and Table 10 in the Results section lists those YAC-containing yeast cell lines used in FISH analysis.

Cell Preparation

• Lymphoblastoid Cell Lines

In order to maximize metaphase yield, lymphoblastoid cell cultures were often synchronized, based on the description given by Yunis in 1976 and modified by Watt & Stephen in Rooney & Czepulkowski (1986).

Cells were incubated in a 0.45 ug/ml methotrexate (amethopterin) solution for 16 to 18 hours at 37°C, which blocked cells at the G1/S interface of the cell cycle. To overcome this block, the media was replaced and thymidine added to a final concentration of 0.24 ug/ml. The cultures were incubated at 37°C for 4 to 5.5 hours, depending on the particular cell line or culture being used. Using several different flasks of the same cell line, the length of incubation with thymidine could be staggered to obtain a sample in which the cells were just entering metaphase. When 15 minutes of the thymidine incubation period remained, Colcemid® (deacetyl methyl colchicine) was added at a final concentration of 0.05 ug/ml and incubated at 37°C to arrest cells in metaphase. After this incubation, the majority of the media was aspirated to remove most of the thymidine and Colcemid®. Fresh media was added and then the contents of the flask transferred to a 15 ml Corning tube and centrifuged at 800 to 1000 RPM for 8 to 10 minutes. The cell pellet was resuspended in 10 ml of hypotonic solution (0.075 M potassium chloride/ KCl), incubated in a 37°C water bath for 10 minutes, and then pelleted at 800 RPM for 8 to 10 minutes The supernatant was removed and the cells gently resuspended in the remaining KCl. Cold fixation solution (3 parts methanol: 1 part glacial acetic acid) was added to the cells up to a volume of 2 ml, followed by the addition of another 8 ml. Centrifugation was again performed, the supernatant removed, and the fixation step repeated two more times. After the third fixation step, the cells were resuspended in a smaller volume (approximately 2 to 4 ml), with a cell density appropriate for the preparation of metaphase spreads for FISH (this could be adjusted later). The cell preparation can be stored at -20°C for several years.

In cases where the synchronization of a lymphoblastoid culture led to preparations with very few metaphase cell spreads, a nonsynchronized method was employed as described by Watt & Stephen in Rooney & Czepulkowski (1986). In this method, the methotrexate and thymidine steps were omitted and a longer exposure to a higher concentration of Colcemid® was required, resulting in a general shortening of the chromosomes. Colcemid® was added to a final concentration of 0.2 ug/ml and incubated at 37°C for one to two hours. The Colcemid® must be left in for a longer period of time in order to arrest enough cells in metaphase.

After the incubation period, the cells were treated as described in the synchronized procedure.

- **Fibroblast Cultures**

This procedure is a combination of information regarding time and concentration of incubation with Colcemid® obtained via personal communication with C. Lee, D. M'Fadyen, and B. Sellinger.

Due to the slower division rate of fibroblasts, Colcemid® was left in the culture medium for a longer period of time at 37°C (anywhere from 1 to 4 hours) at a concentration of 0.1 ug/ml. The cells were monitored periodically under the microscope to determine the mitotic index. Those cells undergoing mitosis appeared "balled up". Once the mitotic index reached approximately 50%, the cells were trypsinized and processed as described above. The cells of patient CM20 were prepared for FISH analysis in this manner, however after 4 hours in the Colcemid® solution, the mitotic index was only about 10%. The cells were processed at this stage as described above for lymphoblastoid cells and yielded an adequate cell preparation for metaphase analysis.

Slide Preparation

The ice-cold cell suspension was dropped from waist height dropwise onto cold, wet, grease-free slides placed at a 30° to 45° angle (Rooney & Czepulkowski, 1986). Slides were rinsed with several drops of cold fix solution to attempt to remove cell membranes and other debris. The slides were placed on a platform suspended above a water bath at 70 to 80°C to facilitate the spreading of the chromosomes while drying. Slides were examined under the microscope to locate the best area for hybridization and marked with a diamond pen on the reverse side of the slide. There is some debate about the optimal age of slides for FISH, and about storage conditions of the unFISHed slides. I found that very fresh slides resulted in fuzzier chromosomes, therefore I normally aged slides for one week. I have however used slides that were 6 months old as well

as some freshly dropped ones that had been stored at room temperature that worked well.

Probe Preparation

- **Biotinylation**

Various types of DNA were used in the preparation of probes for FISH. Regardless of the type used, the labeled probe should be in fragments of sizes 50 to 500 bp (according to the BRL kit description), with the majority between 150 to 250 bp (Lichter et al., 1988) for optimal hybridization. DNA was labeled using the BRL Bionick™ labeling system.

Biotinylation of cosmid DNA was carried out as described in the BRL Bionick™ labeling system protocol with some modifications. Briefly, the procedure was as follows: 1 to 2 ug of DNA (prepared either by alkaline lysis miniprep or by the Qiagen maxiprep procedure) was combined with 5 ul of dNTP mixture containing biotin-14-dATP, 5 ul of enzyme mix containing DNA polymerase I and DNase I, and water to a final volume of 50 ul. This mixture was incubated at 15 to 16°C in a Styrofoam box for approximately two hours. The purpose of the lower temperature was to limit DNase I activity.

After incubation, EDTA was added at a concentration of 27.3 mM to stop enzyme activity. The DNA was then precipitated by the addition of 1/10 the reaction volume of a 3 M sodium acetate solution and two volumes of 95% EtOH and placed at -70°C for 30 minutes. The ethanol was decanted following a 10 minute centrifugation at 14,000 x g. To eliminate some of the salt, 70% EtOH was added, mixed well, placed at -70°C for 20 minutes, centrifuged for 5 minutes at 14,000 x g, decanted and then dried under vacuum with the top of the tube covered with punctured parafilm until completely dry (about 8 to 10 minutes). The biotinylated DNA was resuspended in 100 ul of TE and stored at -20°C for up to one year.

Biotinylation of total yeast DNA containing human-derived YACs required more DNA (approximately 3 ug, B. Barnoski, personal

communication) because the YAC represents only about 5% of the total yeast DNA (Chumakov et al., 1992a). Due to the larger size of the YAC DNA (100-2000 kb) compared to cosmid DNA (approximately 40 kb), the biotinylation reaction was allowed to proceed for a longer period of time. Generally, the reaction was allowed to proceed 6 hours and then an aliquot was subjected to agarose gel electrophoresis, as suggested by B. Barnoski, to determine if the fragments were in the size range optimal for FISH. If so, the reaction was stopped and prepared as above.

- **Probe Preannealing**

Approximately 100 to 200 ng of biotinylated cosmid DNA (1/10 of the resuspended volume) was combined with competitor DNA (Cot^{-1} DNA from BRL). Cot^{-1} DNA is obtained from sheared human placental DNA enriched for repetitive sequences. Normally, the first time a test probe was used, it was combined with a control probe (that was also biotinylated) to confirm its presence on chromosome 22. For one probe, 0.6 ug/ml of Cot^{-1} DNA was used and for two, 1.2 ug/ml (M.A. Riazi, personal communication).

Competitor DNA and labeled probe(s) were combined and precipitated with 1/10 volume 3 M sodium acetate and two volumes 95% EtOH as described in the information accompanying BRL human Cot^{-1} DNA. The resultant pellet was washed with 70% EtOH and vacuum dried as described above. DNA was resuspended in a 50% deionized formamide, 2X SSC, 20% dextran sulfate solution with a total volume of 15 ul by vortexing and placing at 37°C for several hours to overnight to enable complete resuspension. When biotinylated total yeast DNA containing human-derived YACs was used as a probe, the procedure varied slightly: approximately 500 ng of biotinylated probe was combined with competitor DNA. In this case, both Cot^{-1} (final concentration of 0.1-0.6 mg/ml) and sheared herring sperm DNA (final concentration of 1.3 mg/ml) were used. The DNA pellet was resuspended in twice the normal volume to make resuspension of the greater than normal quantity of DNA easier (B. Barnoski, personal communication).

Following resuspension, the biotinylated probe and competitor DNAs were denatured and partially preannealed to compete out any repetitive sequences before addition to the slide. To denature, the probe was placed in a 75 to 80°C water bath for 5 to 10 minutes and then immediately placed back at 37°C for approximately one hour (the time varied from one hour to 1.5 hours with different probes used) to preanneal (Lichter et al., 1988).

Hybridization

Prior to the addition of biotinylated probes to slides, the slides were treated with RNase A and denatured as described in the ONCOR in situ hybridization leaflet. Briefly, the slides were incubated at 37°C with 100 ug/ml of RNase A for 30 to 60 minutes. After this incubation, the slides were washed in 2X SSC, dehydrated in an ethanol series (70%, 95%, 99%) and air dried. To denature the chromosomes, a maximum of two slides at a time were immersed in a 70% formamide, 2X SSC solution at 70°C for one slide and 71°C for two slides for 2 minutes and then dehydrated as before.

Dry slides were preheated to 37°C. The probe hybridization mixture was added, a coverslip applied, and sealed with rubber cement. The slide was placed overnight (16 to 18 hours) in a humidifying chamber at 37°C (depicted in Figure 8 and previously described in Mears 1995). Sometimes, double hybridizations were carried out on the same slide (two coverslips positioned relatively close together but sealed separately to prevent mixing of the probe/hybridization mixtures) in order to perform two hybridization experiments at once. These experiments worked well and were an effective time saver.

Figure 8. Humidifying chamber used in FISH. A slide and coverslip were supported by a platform constructed of two stacks of four to six microscope slides taped together. On the bottom of this plastic chamber was a moistened piece of 3MM Whatmann to prevent the hybridizations from drying out. As an extra precaution, the dish was wrapped in saran wrap to prevent loss of moisture in the overnight incubations.

Post-Hybridization

- **Washes**

After hybridization, washes at 42 to 45°C (depending on the probe being used) were performed as described in Kuwano et al (1991) with the following modifications: 15 minutes in 50% formamide/2X SSC, 15 minutes in 50% formamide/1X SSC, 2X 10 minutes in 1X SSC, 5 minutes in 0.1X SSC. Another wash in 0.1X SSC was performed at room temperature, followed by a 2 minute rinse in BT-NaCl (a mild detergent composed of 0.1 M sodium bicarbonate, 0.3 M sodium chloride and 0.05% Triton X-100).

- **Detection and Amplification**

Detection and amplification were performed as described in the ONCOR manual except that blocking reagent one was a 3% BSA (BRL)/ 4X SSC solution and blocking reagent two was a 1/10 goat serum (SIGMA)/ 4X SSC solution (B. Sellinger, personal communication). Briefly, the procedure was as follows: a 5 minute room temperature incubation with blocking reagent one on the slide covered with a full sized plastic coverslip was performed, followed by a 30 minute 37°C incubation in a humidifying

chamber with avidin-FITC (fluorescein isothiocyanate) from ONCOR. Washes were then performed at 45 to 48°C in a solution of BT-NaCl. Next, a 5 minute room temperature incubation with blocking reagent two was performed followed by a 20 minute 37°C incubation with antiavidin (ONCOR) and then washes as described above. Another avidin step was then performed to amplify the signal.

- **Counterstaining and Photography**

Slides were counterstained with a solution of propidium iodide and antifade (ONCOR), covered with a glass coverslip, sealed with nailpolish and visualized using a Zeiss Axiophot photomicroscope with a Zeiss 9 filter transmitting wavelengths 450 to 490nm. Selected metaphase spreads were photographed using Kodak Ektachrome Elite 100 color slide film. Slides were stored at -20°C protected from light.

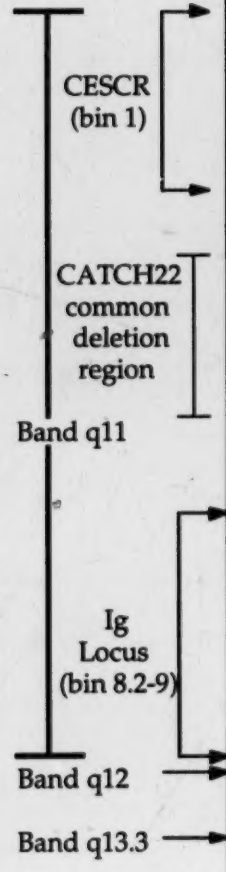
- **Reamplification**

Detection of weak signals sometimes required reamplification. This was avoided in most cases due to the accompanying increase in background signal. The procedure used was based on and modified from that of M.A. Riazi and B. Sellinger (personal communication). Coverslips were removed carefully and the slide washed in 2X SSC for 2 minutes at room temperature, sterile water for 2 minutes at room temperature, and then BT-NaCl at 45°C for 2 minutes. Amplification steps were then carried out, consisting of an antiavidin incubation followed by an avidin incubation, as outlined above. An extra wash after incubation with the antiavidin and avidin were performed to help reduce the background signal.

Table 8. Probes used for quantitative dosage and RFLP analysis. The loci are listed in a proximal to distal order on chromosome 22q. "D22S" denotes a single copy (S) locus on chromosome 22. The capital letters in the "insert" column denote enzymes used to release the insert. R= Eco RI, H= Hind III, T= Taq I. The number following the letters are the size of the insert in kilobases. The cosmid column indicates the "address" of a cosmid isolated with the insert fragment from the chromosome 22 specific cosmid library LL22NC03 (de Jong et al., 1989) The cosmids were screened and characterized by myself, M. Budarf or A. Johnson. The polymorphism column indicates the restriction endonuclease used to digest the genomic DNA, the designations "A" and "B" refer to an allele of the polymorphism, and the number in brackets indicates the size of the allele in kilobases. "Constants" refers to fragments present in all individuals and are thus not polymorphic.

Locus	Probe	Insert	Cosmid	Polymorphism	Reference
Test Probes:					
D22S9	p22/34	R/H 1.8	N107D6	Taq I A1(5.8) A2(3.2)	McDermid et al., 1985
D22S43	H32	H 1.5	N41A6/A7	Taq I A1(4.8) A2(3.8) A3(2.9)	Budarf et al., 1991
D22S57	H98	H 0.7	---	BstXI A1(2.6) A2(2.2) Msp I B1(2.5) B2(1.5)	Budarf et al., 1991
D22S181	NB17	R/H 0.7	N54G12	Taq I A1(2.9) A2(2.2)	Lekanne-Deprez et al., 1991
D22S427	---	R 1.1 R/H 0.85	N106E4	-----	Gyapay et al., 1994, Collins et al., 1995, Budarf & McTaggart unpublished
D22S36	H11	H 0.9	N103A2	Msp I A1(3.3) A2(1.6) constants (3.7, 2.3)	Budarf et al., 1991
D22S75	N25	H 2.4	N5D9	Taq I A1(5.8) A2(3.2)	McDermid et al., 1989
Control Probes:					
D21S110	p21-4U	H 3.0	---	-----	Spinner et al., 1989
D21S15	pGSE8	T 1.9	---	-----	Stewart et al., 1985
D22S45	pH41a	H 1.4	---	-----	Budarf et al., 1991

Table 9. Probes used in FISH analysis. Loci are ordered proximal to distal on 22q (based on maps in Collins et al., 1995; Kawasaki et al., 1995; Frippiat et al., 1995). Although the relative locations of the probes are known, the distances are not. Plasmids are denoted by a "p" in front of the probe name or clone. The cosmids are denoted by an "N" in front of the address (the "address" is the location of the cosmid clone in a gridded chromosome 22-specific library, LL22NC03, of de Jong et al., 1989). The addresses were provided by M. Budarf, A. Johnson, myself, or in the references listed here. To the left of the table are important reference points such as the chromosomal location (cytogenetic band and somatic cell hybrid bin) as well as landmark areas such as the cat eye syndrome critical region (CESCR) and the CATCH22 common deletion region, and the immunoglobulin locus (Ig locus).



LOCUS (PROBE)	CLONE USED FOR FISH	REFERENCE(S)
-------	N64E9	Collins et al., 1995
D22S111 (KI-197)	N39C5	Carey et al., 1990
D22S50 (H74)	N80D1	Budarf et al., 1991
D22S9 (22/34)	N107D6	McDermid et al., 1985
D22S43 (H32)	N41A6/N41A7	Budarf et al., 1991
D22S181	N54G12	Lekanne-Deprez 1991
D22S427	N106E4	Gyapay et al., 1994
D22S36 (H11)	N103A2	Budarf et al., 1991
D22S75 (LN25)	N5D9	McDermid 1989
D22S788 (LN41)	N68A1	Budarf 1994 (GDB#374864)
-------	N92D4	Budarf, unpublished
HCF2	N17D9	Blinder et al., 1988
CRKL	pCRKL[1]	ten Hoeve et al., 1993
EN34	phage EN34, N94C3, N110A8	McDermid, unpublished
D22S112 (KI-205)	N19A11	Carey et al., 1990
-------	N11E6	Kawasaki et al., 1995
-------	N118F11	Kawasaki et al., 1995
G12-1	phage G12-1	McDermid & Emanuel 1994; Frippiat et al., 1995
VpreB	N90D11	Frippiat et al., 1995
-------	N52E9	Kawasaki et al., 1995; Frippiat et al., 1995
-------	N61E11	Kawasaki et al., 1995; Frippiat et al., 1995
-------	N14C6	Kawasaki et al.,1995; Collins et al., 1995
-------	N102B11	Kawasaki et al., 1995; Frippiat et al., 1995
ADORA2A (pHUAD2)	N30D1	Libert et al., 1989; McDermid et al., 1993; MacCollin et al., 1994
D22S39 (H17)	N108A7	Budarf et al., 1991

RESULTS

PHYSICAL MAPPING

♦ YAC contig of the CESCR

An important prerequisite to the study of genes involved in cat eye syndrome (CES) is cloning of the CESCR, which is being done as a collaboration between a number of research groups. As part of this effort, M^{c}Dermid et al (1996) constructed a YAC contig of this region. YACs as a vector system were chosen because of their large size. The average insert size in the CEPH MegaYAC library is 1.0 Mb with a size range of 0.1Mb to 2Mb (Chumakov et al., 1992a & 1995). One serious problem with YACs, however, is chimerism: they may be composed of fragments from different chromosomes or non-contiguous fragments from the same chromosome. Generally, the rate of chimerism is 30-50% (Chumakov et al., 1992a, 1992b, 1993, 1995; Cohen et al., 1993).

My role in this project was to test the YACs isolated by others for chimerism. One simple method of detection is fluorescent *in situ* hybridization (FISH) analysis. Two approaches could have been taken. PCR amplification of the YAC-containing yeast DNA using Alu primers could be used to generate human-specific products from the YAC. These products could subsequently be used as probes for FISH analysis of human metaphase chromosomes. One concern with this method is incomplete representation of the entire human portion of the YAC as Alu PCR products represent only about 1% of the YAC sequence (Chumakov et al., 1992a). There is thus a concern that chimerism could remain undetected. Therefore, a second method involving biotinylation of total yeast genomic DNA from YAC-containing strains was used, ensuring representation of the entire cloned human sequence. Non-specific background hybridization due to excess yeast DNA was a concern as a YAC accounts for only about 5% of the total yeast DNA (Chumakov et al., 1992a). To suppress background hybridization, the biotinylated total yeast DNA was

preannealed with herring sperm DNA. Hybridization to human repetitive sequences was prevented by preannealing with human Cot^{-1} DNA.

Of the 15 YACs used in the FISH chimerism analysis, 11 were chimeric (73%). This rate of chimerism is higher than the expected 30-50% however the chimerism rate can vary in different parts of the genome (Chumakov et al., 1995) and our sample size was relatively small and thus may not be representative. Table 10 lists the YACs tested for chimerism and results of the FISH analysis. Listed are the chromosomes on which hybridization was observed. In most cases, the specific chromosomes could not be determined because banding was not done, however the size of the chromosome and placement of the centromere enabled determination of chromosome group (Group A= chr. 1, 2, 3; Group B= chr. 4, 5; Group C= chr. 6, 7, 8, 9, 10, 11, 12, X; Group D= chr. 13, 14, 15; Group E= chr. 16, 17, 18; Group F= chr. 19, 20; Group G= chr. 21, 22, Y). The table contains some YACs not included in Table 2 from M^{c}Dermid et al (1996) and omits one not tested for chimerism in our laboratory (800A4). To confirm the presence of the YAC on chromosome 22 and within the CESCR, metaphase spreads were prepared from a CES individual possessing a supernumerary CEC. Figure 9 shows FISH photographs from a chimeric and a non-chimeric YAC-containing yeast strain.

With some of the chimeric clones, the relative strengths of hybridization to different chromosomes differed. This variation in signal intensity was judged subjectively and was only noted in cases where the difference was consistent for the greater than 50 metaphases examined for each hybridization.

Table 10. Chimerism of YACs in the CESCR. This table is a modification of Table 2 of M^{c}Dermid et al (1996).

YAC	Size (kb)†		FISH Results
	Expected	Observed	
745G7	1410	640, 860 980, 1200 §	22 (weak) + C (strong) + D (weak)
723B6	790	720	22 (weak) + 2 (strong) + C/E (weak) + D (weak)
715A9	390	1600	22 + B/C
776H2	1490	1280	22 (strong) + 2 (strong) + B (weak) + D (weak)
734B10	n/a	1000	22 (strong) + 21 (weak)
829E11	1140	950	22 + B/C
878D3	1190	1010	22 (weak) + A (strong)
829D11	410	980	22 + B/C
100G7	430	380	22
925G12	860	450, 570 690, 790 §	22 + C
803G9	1390	200 or 580*	22
891F12	480	620	22 (weak) + B/C + D
891C8	120	160	22 (very weak) + D
422G4	n/a	230	22
409G4	n/a	nd	22

n/a= data not available nd= not done

†Expected sizes of the YACs were obtained from information provided in the Whitehead database except for YAC 803G9, in which the expected size is from Morrow et al (1995). Observed sizes were determined by D. Shkolny, A. Johnson & H. M^{c}Dermid by PFGE analysis as described in M^{c}Dermid et al (1996).

§ Multiple YACs in each isolate (see text)

* One YAC per isolate but differing in size between isolates (see text). The 580kb isolate was used in the FISH analysis.

Figure 9. FISH photographs of chimeric YAC 715A9 (a), and non-chimeric YAC 803G9 (b). Metaphase spreads were prepared from CM02, a patient with a CEC. (a) YAC 715A9. Note the signal on both normal chromosomes 22 (long arrows), the CEC (arrowhead), and the distal tip of the long arm of chromosome 2 (short arrows). (b) YAC 803G9 hybridizing to only the two normal chromosomes 22 (arrows) and the CEC (arrowhead).

a

b

- **Map location of locus D22S111 (probe KI-197)**

A second set of FISH experiments was done to clarify a discrepancy in the map position of probe KI-197 (locus D22S111), which is a fragment from a flow sorted chromosome 22-specific library (Dumanski et al., 1991). Budarf et al (1996) assigned KI-197 by PCR to somatic cell hybrid bin 8.1 which is a large distance from the CESCR (bins 1 and 2). This was in disagreement with Collins et al (1995) who placed an STS (sequence tagged site) generated for a portion of the sequence of D22S111 in two different locations, one of which was very close to the centromere of the long arm of chromosome 22 (within the proximal 1 Mb of the CESCR) and the other adjacent to the HCF-2 gene in somatic cell hybrid bin 8.1. PFGE data from H. McDermid indicated that D22S111 (KI-197) did not map to bin 1 (adjacent to the centromere). Figure 10 is a representation of the area of interest constructed from Collins et al (1995) and Budarf et al (1996).

To resolve these discrepancies, FISH analysis was performed using patient CM15. The supernumerary ring chromosome 22 found in this patient was used by Mears et al (1995) to place the distal boundary of the CESCR between the ATP6E gene and locus D22S57. I isolated cosmid N39C5 from the chromosome 22-specific library LL22NC03 (deJong et al., 1989) with the KI-197 probe and confirmed the presence of this 3.2 kb HindIII probe on the cosmid by hybridization analysis. Budarf confirmed the presence of the D22S111 STS on this cosmid by PCR. This cosmid was then used in FISH analysis. Figure 11a demonstrates hybridization of the cosmid to both normal chromosome 22s but not to the supernumerary ring chromosome 22 in metaphases of patient CM15. This indicated the absence of D22S111 in somatic cell hybrid bin 1, supporting Budarf et al's placement of this locus in bin 8.1. The same cosmid was used for metaphase FISH analysis of patient CM11. The absence of the cosmid from this patient's CEC, which includes somatic cell hybrid bins 1 and 2, lends support to the above data. The most likely explanation is that the STS sequence cross-hybridizes with a repeat sequence in bin 1, but the probe and cosmid which contain more sequence than the STS are from somatic cell hybrid bin 8.1. Therefore, both the PFGE data of H. McDermid and the FISH analysis done by myself, demonstrate that locus D22S111 maps to bin

8.1 and not the more proximal location. A small repeat must map to both regions.

A cosmid (N64E9) proximal to locus D22S111 on the map of Collins et al (1995) was also used for FISH analysis on patient CM15 to determine if other probes in this region of the map were affected by repeats. Figure 11b demonstrates the presence of this cosmid on the supernumerary ring chromosome of patient CM15, in agreement with the Collins map. This cosmid also hybridizes to a D-group chromosome, which is not surprising due to the proximity of this region to the centromere and the similarities of the centromeres and pericentromeric regions of the acrocentric chromosomes (Trowell et al., 1993; Waye & Willard, 1989; Kurnit et al., 1984; Gosden et al., 1981; Choo 1990).

Figure 10. Location of probe KI-197 (D22S111), cosmid N39C5 and the STS for KI-197. This map is not to scale and is constructed from Collins et al (1995) and Budarf et al (1996). Above the map are loci and their respective somatic cell hybrid bin locations, cosmids placed on the map of Collins et al., and the CESCR for reference. Below the map are the locations of the probe KI-197, as well as the STS and the cosmid for this probe.

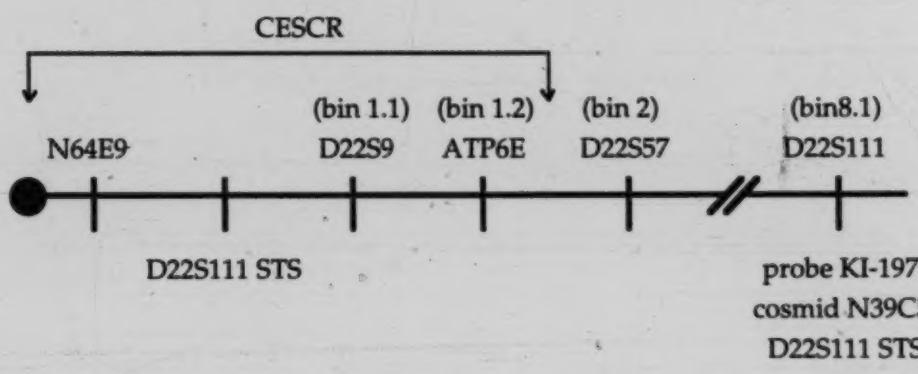


Figure 11. FISH results of cosmid N39C5 (D22S111/KI-197) and cosmid N64E9 using metaphase spreads of patient CM15. (a) Biotinylated cosmid N39C5 and control cosmid N108A7 (locus D22S39 in 22q13.3) demonstrating the absence of hybridization of N39C5 on the supernumerary r(22) which contains somatic cell hybrid bin 1 sequence. The larger arrows identify the normal chromosomes 22 which possess three to four signals. The more terminal signal(s) are from a control probe which unambiguously identifies chromosome 22. The arrowhead iudentifies the unlabelled supernumerary r(22). (b) Biotinylated test cosmid N64E9 hybridized to the supernumerary r(22) (arrowhead), the normal chromosomes 22 (large arrow), as well as a D-group chromosome (small arrow).

a

b

BREAKPOINTS OF CAT EYE CHROMOSOMES (CEC)

The most common type of duplication associated with CES is a supernumerary inverted duplication chromosome 22 which involves two breakpoints within 22q11.2. These CECs were divided into three types by Mears (1995) based on the extent of the duplication. Classification of these chromosomes was based on the localization of the two breakpoints involved in their formation. Both breakpoints of the type I, or smallest CECs, were localized between the loci D22S181 and D22S36, an interval spanning approximately 1 Mb (McDermid et al., 1996). Mears demonstrated that the type IIa CECs (CM02 and CM03) patients were clearly asymmetrical, with one breakpoint located in the type I interval and the other within or beyond the CATCH 22 common deletion region. Both breakpoints involved in the formation of the type IIb CEC (patient CM01) were within or beyond the CATCH 22 region (Mears et al., 1994). It was thus unknown if the breakpoints located beyond locus D22S36 were clustered. Further studies were necessary to delineate the breakpoints of these three types of CECs. A general depiction of these three types of CECs is shown in Figure 12.

- **Patient Phenotype**

Table 11 summarizes all of the reported phenotypic features of the CES patients utilized in this analysis of CECs. Note that some of the clinical evaluations were done at an early age, making it difficult to ascertain the developmental status of the individual, and that some of the clinical reports were incomplete.

- **Localization of type I breakpoints**

Mears et al (1994) demonstrated that the breakpoints involved in the formation of eight Type I CECs were located between loci D22S181 and D22S36. According to McDermid et al (1996), the estimated distance

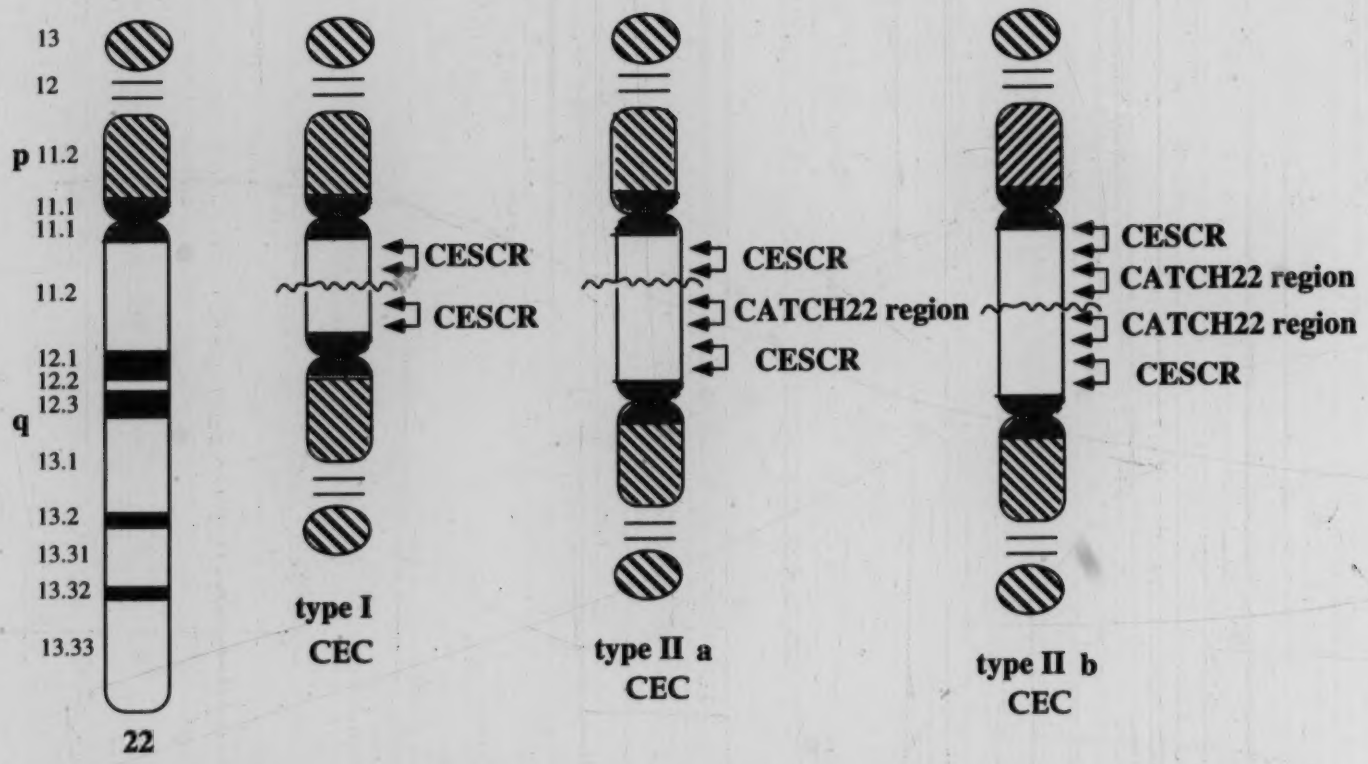


Figure 12. Ideogrammatic representation of a normal chromosome 22, a type I CEC, a type IIa CEC, and a type IIb CEC (modified from Mears 1995). Shown on the chromosomes are the CESCR and the CATCH22 deletion region distal to the CESCR.

Table 11. Phenotypic summary of the patients utilized in this study based on information provided in clinical reports. A (+) or (-) sign indicates the presence or absence, respectively, of a characteristic in the clinical report. A (-) sign thus only indicates the lack of report of a characterisitic in the clinical report and not its absence from that individual. In the case of mental retardation and developmental delay, a question mark (?) indicates no mention of mental and physical development in the clinical report. The percentage of individuals manifesting each trait is given at the bottom of the table, however, because some of the clinical reports are incomplete, these percentages are only estimates. Percentages may not correspond to those given in the introduction due to the small sample size listed here. All of the patients with the exception of CM17 and CM20 have been previously studied in Mears et al (1994), Mears et al (1995), and Mears (1995).

The CHDs listed in the table are as follows: pda= patent ductus arteriosus; tapvr= total anomalous pulmonary venous return; asd= atrial septal defect; murmur= heart murmur (not determined); tof= tetralogy of Fallot; raa= right aortic arch; vsd= ventricular septal defect. Anal anomalies included anal atresia (CM02, CM04, CM07, CM10 and CM11), and anal atresia with fistula (CM01, CM05, CM06, CM08 and CM20). Renal anomalies included hydronephrosis (CM06 and CM20), ureteral reflux and recurrent infections (CM11), and unilateral kidney agenesis (CM06). Renal system examination was not performed in patient CM03. Genital anomalies included hypogenitalia (CM04 and CM08), undescended left testis (CM02), and rudimentary fallopian tubes, uterus hypoplasia, and vagina agenesis (CM06). Skeletal anomalies included Wormian bones and large fontanelles (CM03), thoracic scoliosis and funnel chest (CM05), thoracic kyphoscoliosis, bilateral partial cutaneous syndactyly, and fusion of the eleventh and twelfth vertebrae (CM06), and flattened occiput, depressed maxilla, and short terminal phalanges (CM10). Palatal anomalies included high arched palates (CM04 and CM10).

Feature \ Patient (age*)	CM01 (4 mo)	CM02 (2.5 yr)	CM03	CM04 (4 yr)	CM05 (15 yr)	CM06 (30 yr)	CM07 (31 yr)	CM08 (6 yr)	CM09 (28 yr)	CM10 (adult)	CM11 (1-2 yr)	CM17 (7 mo)	CM20 (13 mo)	%
Ocular coloboma	-	-	+	+	+	+	-	-	+	+	+	-	-	54
Preauricular skin tags/pits	+	+	+	+	+	+	+	+	+	-	+	+	+	92
Anal anomalies	+	+	-	+	+	+	+	+	-	+	+	+	+	85
Congenital heart defects	-	-	pda	-	-	tapvr/asd	-	murmur	tof/raa	-	vsd	tapvr	tapvr/vsd	54
Downslanting palpebral fissures	+	-	+	+	+	+	-	-	-	-	-	+	+	54
Hypertelorism	-	-	-	+	+	+	-	-	-	-	+	+	-	38
Ear anomalies	+	-	-	+	+	-	-	-	-	-	-	-	-	23
Micrognathia	+	-	-	+	-	-	-	-	-	-	-	+	+	31
Renal anomalies	-	-	?	-	-	+	-	-	-	-	+	?	+	23
Genital anomalies	-	+	-	+	-	+	-	+	-	-	-	-	-	31
Skeletal anomalies	-	-	+	-	+	+	-	-	-	+	-	+	-	38
Epicanthal folds	+	-	-	-	+	+	-	-	-	-	-	+	+	38
Palatal anomalies	-	-	-	+	-	-	-	-	-	+	-	+	-	23
Developmental delay/ mental retardation	?	-	?	severe	mild	mild	mild	mild	?	+	-	?	mild	nc

*Age at the time of the last clinical examination

between these two loci from PFGE is about 1 Mb. A new locus, D22S427, mapped between these two loci, based on information from D. Driscoll (Children's Hospital of Philadelphia). D22S427, or AFM288we5, is a polymorphic PCR-amplified dinucleotide repeat found by J. Weissenbach (Collins et al., 1995). Based on PFGE analysis by H. McDermid & myself, this locus was placed about halfway between the loci D22S181 and D22S36. D22S427 maps to the same NotI fragment as D22S181 and the same 650kb NruI fragment as D22S36, thus placing it about 450kb to 650kb from either locus (McTaggart et al., in preparation). The PFGE map in Figure 13 is Figure 3 from McDermid et al (1996) with the D22S427 locus placed on it. D22S427 was used to attempt to reduce the size of the interval containing the CEC type I breakpoints.

A cosmid address for locus D22S427 (N106E4) from the chromosome 22-specific library LL22NC03 was provided by M. Budarf (Children's Hospital of Philadelphia). The presence of this cosmid by FISH analysis on all eight of the type I CECs tested (CM04, CM05, CM06, CM07, CM08, CM09, CM10, CM11), indicated that at least one of the two breakpoints involved in the formation of these CECs was located between D22S427 and D22S36. Figure 14 is a photograph showing the FISH results for one of these individuals. Unfortunately, determination of the presence of one or two copies of D22S427 on each CEC was not possible from the FISH results. The proximity of the two breakpoint regions did not allow the resolution of two signals and dosage differences in signal intensities were not consistent enough to make any conclusions. Quantitative dosage analysis was thus necessary. Unique restriction fragments from cosmid N106E4 were selected by hybridization of a number of different restriction fragments from the cosmid to a Southern blot containing human genomic DNA digested with various restriction endonucleases. Two restriction fragments, both resulting in the same hybridization pattern, were selected (see Table 8 in Materials and Methods).

Figure 13. Physical mapping of locus D22S427 showing a PFGE restriction map from McDermid et al (1996) to which D22S427 (*) has been added. Restriction enzymes Not I and Nru I were used to place D22S427 between D22S181 and D22S36. Above the map are the locations of loci in the region. Loci beginning with "S" should be prefaced by "D22".

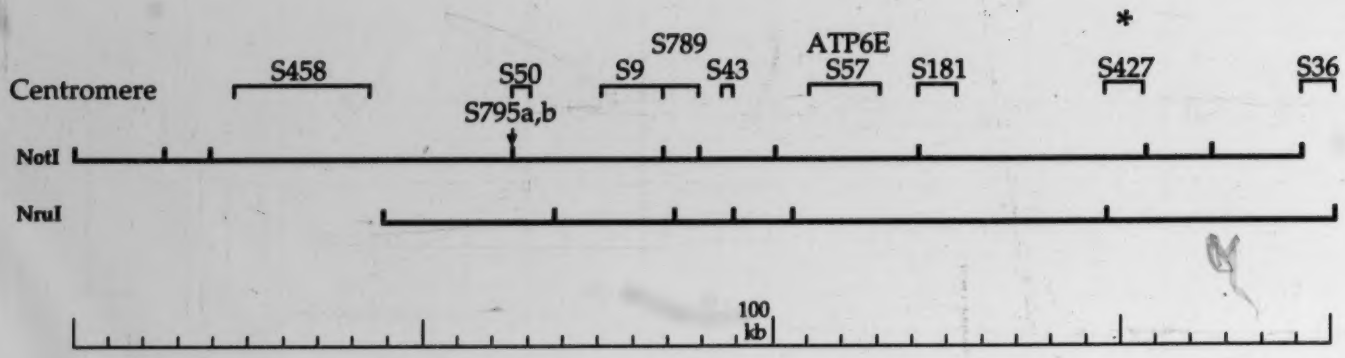


Figure 14. FISH results of patient CM07 hybridized with biotinylated cosmid N106E4 (locus D22S427) and control cosmid N108A7 (locus D22S39 in 22q13.3). Chromosome 22 is identified by the arrows and the CEC by the arrowhead. The normal chromosomes 22 display three to four signals; the one to two most terminal represent the control cosmid.

Quantitative dosage analysis was performed as described previously in Mears et al (1994) and in the Materials and Methods.

Table 12 summarizes the results of dosage analysis of locus D22S427 for the type I patients tested. Due to some problems experienced with the quantitative dosage analysis, these experiments were subsequently repeated by H. M^{c}Dermid. The results of the analysis performed by H. M^{c}Dermid and myself demonstrated the presence of four copies of locus D22S427 in all of the type I individuals tested. In all cases with the exception of CM11, this indicates the presence of two copies of D22S427 on the CEC, indicating both breakpoints involved in the formation of the CEC occur within the D22S427-D22S36 interval.

The presence of four copies of the locus D22S427 in CM11 does not necessarily indicate the presence of two copies of D22S427 on the CEC because of the presence of an interstitial duplication encompassing locus D22S36 (Mears et al., 1994). FISH analysis demonstrated the presence of at least 1 copy of D22S427 on the CEC, however the fourth copy shown by dosage analysis could be present on the chromosome 22 containing the interstitial duplication. Subsequently, dosage analysis performed by H. M^{c}Dermid on YM11, the father of CM11, who also possesses an interstitial duplication of D22S36 (Mears 1995), demonstrated the presence of only two copies of D22S427. Thus, the interstitial duplication encompasses only locus D22S36 and therefore both extra copies of D22S427 in patient CM11 are located on the CEC.

The CEC of a new patient (CM17) was characterized and is included in Table 12. Quantitative dosage analysis revealed two copies of locus D22S36, indicating a type I CEC. Figure 15 is a portion of an autoradiograph from a dosage experiment with patient CM17. The locus being tested in this case was D22S9, one of the more proximal loci in the CESCR. Figure 16 contains the raw densitometric values, ratios and comparisons between individuals using the Wilcoxon rank sum test. The result of this analysis is that locus D22S9 is present in four copies in this patient, which is the expected result in individuals with a CEC. RFLP analysis was used to determine the parent-of-origin of the duplication. The only informative

polymorphism was that for locus D22S181 which revealed maternal origin. Figure 17 is a portion of an autoradiograph showing the results obtained for CM17, XM17, and YM17.

Some of the dosage values for locus D22S427 that I performed were uncertain and therefore classed as preliminary results. In all of the analyses, normal and trisomic control individuals were used to ensure that a statistically significant difference between two and three copies was detectable. If the ratios for these two controls were not significantly different, all of the values should be discarded; however this happened so frequently that some inter-patient analysis was attempted. Problems that plague dosage analysis include variation in Southern transfer and hybridization, and limitations in film sensitivity. This may account for some problems encountered in the study. I believe the most likely explanations are uneven Southern transfer and differences in the intensity of hybridization signal between the control and test fragments. The control probe consistently labeled much more efficiently and thus resulted in a much more intense signal than the test probe. Retrospectively, attempts should have been made to equalize the activity levels of the two probes.

Table 12. Copy number of locus D22S427 in type I CES patients. The number represents the number of copies of the locus in each individual. The non-shaded values can be found in Mears et al (1994) and are included here as relevant background information. The values for D22S427 were calculated for all individuals except CM04 who demonstrated heterozygosity of a polymorphism for the probe used. CM07 and CM08 are related and thus included in the same column. Also included are the dosage results for CM17, a new patient.

Patient / Locus	CM04	CM05	CM06	CM07 CM08	CM09	CM10	CM11	CM17
D22S57	4	4	4	4	4	4	4	4
D22S181	4	4	4	4	4	4	4	nd
D22S427	nd	4*	4*	4*	4*	4*	4	4
D22S36	3	2	2	2	2	2	3	2
D22S75	2	2	2	2	2	2	2	2

nd = not done

*The copy number of locus D22S427 for these patients are uncertain. The values were obtained by comparison to other individuals with a type I CEC present on the same Southern blot because the 2 and 3-copy controls were not significantly different. These preliminary results were later confirmed by H. M^{c}Dermid for CM05, CM06, CM07, CM09, CM10.

Figure 15. One replicate of an autoradiograph used to determine the copy number of D22S9 for CM17. One of six replicates from a Southern blot of DNA from CM17, GM03657 (2-copy control), and GM02325 (3-copy control) digested with HindIII restriction endonuclease. The blot was hybridized with the test probe D22S9 (5.8kb) and the control probe D21S110 (3.0kb). The autoradiograph was scanned using a BIO-RAD GS-670 densitometer and the signals measured using Molecular Analyst version 1.4 software. These values and their analysis using the Wilcoxon rank sum test are shown in Figure 16.

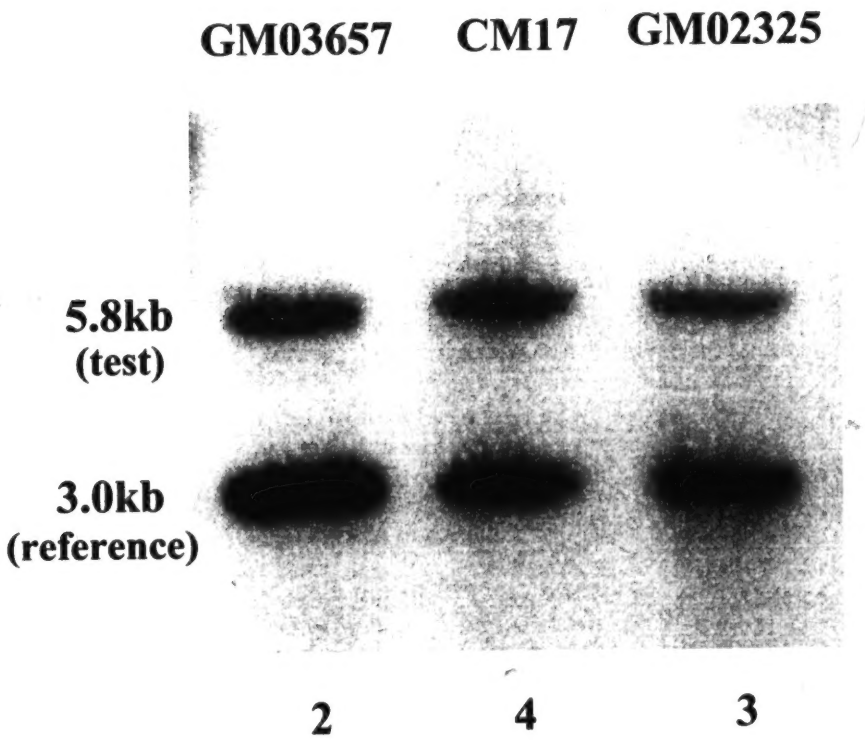

GM03657
CM17
GM02325
5.8kb
(test)
3.0kb
(reference)
2
4
3

Figure 16. Copy number determination of locus D22S9 for patient CM17.

(a) The raw densitometric values (test/control column) as well as the ratios of the test to control signal are given for all six replicates of CM17, GM03657, and GM02325.

(b) Statistical analysis of the ratios calculated in (a). The ratios for each individual are compared to each of the other two, and given a "rank" value. The sums of these ranks are calculated and then the significance level determined from a Wilcoxon table. The P-value (probability value) is given below each comparison. The P-value represents the the probability that the conclusion reached is false as a result of chance. The inter-individual comparisons reveal that all three individuals demonstrate significant difference from eachother, indicating CM17 has four copies of locus D22S9.

a

Individual	Test/Control	Ratio	Individual	Test/Control	Ratio	Individual	Test/Control	Ratio
GM03657	0.51/1.37	**0.372**	**CM17**	0.46/0.76	**0.605**	**GM02325**	0.27/0.60	**0.45**
	0.58/1.54	**0.377**		0.45/0.80	**0.563**		0.42/0.74	**0.568**
	0.54/1.50	**0.36**		0.57/0.86	**0.663**		0.33/0.61	**0.541**
	0.45/1.32	**0.341**		0.53/0.97	**0.546**		0.29/0.73	**0.397**
	0.34/1.04	**0.327**		0.41/0.74	**0.554**		0.39/0.89	**0.438**
	0.43/1.02	**0.422**		0.48/0.78	**0.615**		0.21/0.55	**0.382**

b

GM03657		CM17	
Ratio/Rank		Ratio/Rank	
0.372	4	0.605	10
0.377	5	0.563	9
0.36	3	0.663	12
0.341	2	0.546	7
0.327	1	0.554	8
0.422	6	0.615	11
Rank Sum	**21**	**Rank Sum**	**57**

CM17>2 copies
(P=0.001)

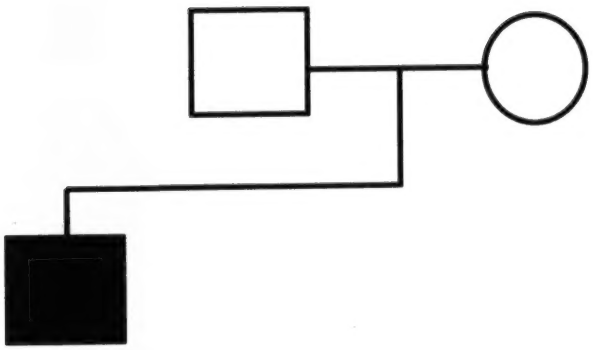
GM03657		GM02325	
Ratio/Rank		Ratio/Rank	
0.372	4	0.45	10
0.377	5	0.568	12
0.36	3	0.541	11
0.341	2	0.397	7
0.327	1	0.438	9
0.422	8	0.382	6
Rank Sum	**23**	**Rank Sum**	**55**

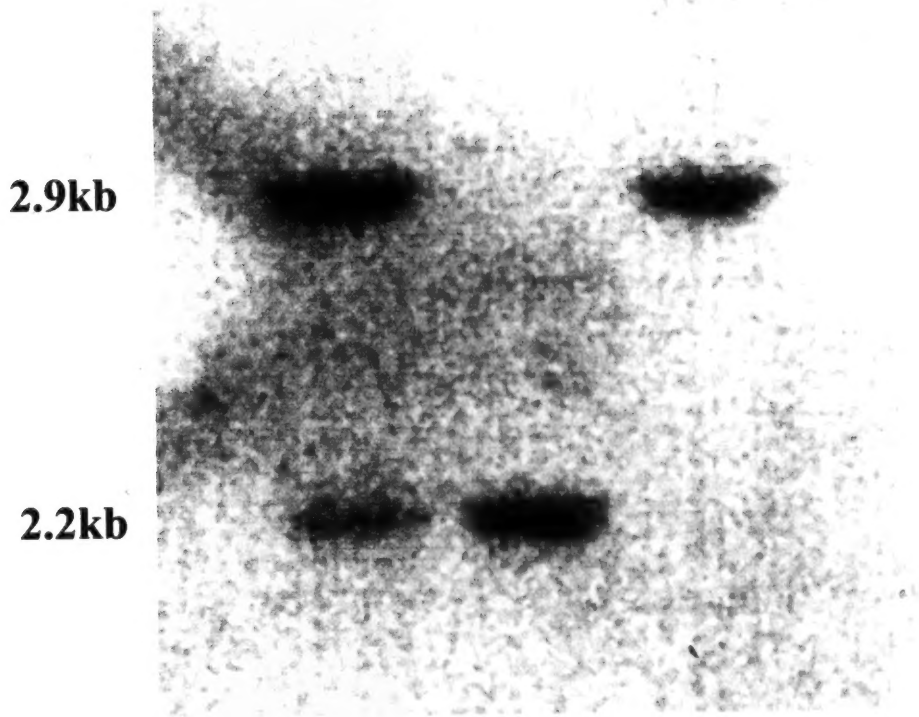
2 copies<3 copies
(P<0.001)

CM17		GM02325	
Ratio/Rank		Ratio/Rank	
0.605	10	0.45	4
0.563	8	0.568	9
0.663	12	0.541	5
0.546	6	0.397	2
0.554	7	0.438	3
0.615	11	0.382	1
Rank Sum	**54**	**Rank Sum**	**24**

CM17>3 copies
(P=0.005)

Figure 17. RFLP analysis of CM17, YM17, and XM17 to determine the parent of origin of the duplication of CM17. DNA was digested with TaqI restriction endonuclease and probed with a 0.7kb HindIII/EcoRI restriction fragment representing locus D22S181. CM17 is polymorphic for this locus, possessing both the 2.9kb and 2.2kb fragments. The maternal 2.9kb fragment shows greater intensity than the paternal 2.2kb fragment, indicating maternal origin.




2.9kb
2.2kb

- **Localization of type IIa and type IIb CEC breakpoints**

Mears et al (1994) reported three individuals with larger CECs (CM01, CM02, and CM03). Two of these (CM02 and CM03), were referred to as type 2 which I refer to as type IIa. The proximal breakpoint was located in the D22S181 to D22S36 interval and the distal breakpoint was somewhere distal to locus D22S75. Thus the CECs of these individuals were clearly asymmetric. The CEC of the third of these individuals, CM01, was classified as a type 3 marker (Mears 1995) which I now denote as type IIb. Both breakpoints involved in the formation of this CEC were distal to locus D22S75. Since this time, another patient, CM20, has been added to the type IIa group. Dosage analysis demonstrated three copies of loci D22S36 and D22S75, indicating that the duplication extended beyond the type I interval. I later confirmed this with FISH. RFLP analysis was performed to determine the parent-of-origin of the duplication, however it was uninformative for all of the polymorphisms used (loci D22S9, D22S57, D22S181, D22S36, and D22S75).

The purpose of my experiments with these patients was to clarify the proximal breakpoint of the type IIa CECs with respect to locus D22S427, and to localize the distal breakpoints of both the type IIa and type IIb CECs.

A summary of the phenotypic features of these patients can be found in Table 11. FISH analysis using cosmid N106E4 for locus D22S427 was not performed in these individuals as this would not have been informative. Copy number using FISH signal intensity cannot be determined on these CECs because the proximity of the signals results in the appearance of one signal. Therefore, determination of copy number would have to be based on signal intensity which is not reliable with our equipment. Quantitative dosage analysis was thus performed to clarify the position of the proximal breakpoints of the type IIa CECs. The proximal breakpoints in CM02, CM03, and CM20 were localized to the D22S427-D22S36 interval (H. M^{c}Dermid & myself). Both breakpoints of CM01 are located somewhere beyond locus D22S75 (Mears et al., 1994).

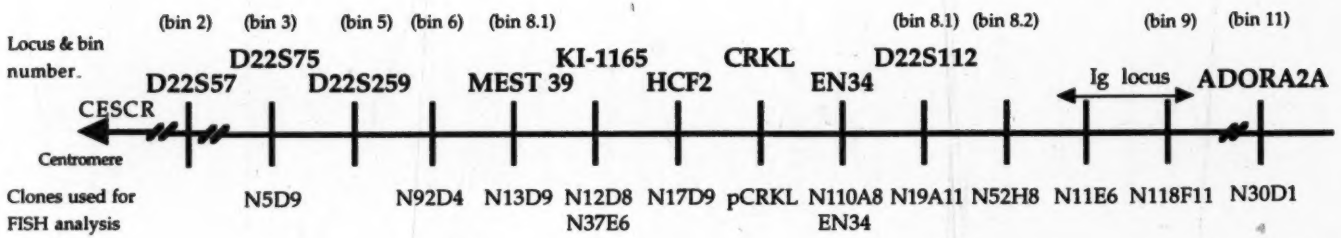
Distal breakpoints of the type IIa and type IIb CECs were localized using FISH analysis. Figure 18 is a map of the region with the relative locations of the loci and clones tested, as well as the results for each patient. Not all of the clones were tested on all four patients. Figure 19 has photographs of some of the FISH results. Interestingly, the distal breakpoints of CM01, CM02, and CM20 are located in the same interval and the breakpoint of CM03 is located just distal to this region. The phage clone EN34 was the first probe tested that differentiated CM03 from the others (Figure 19b and 19c). The next most proximal probe, pCRKL (Figure 19a), was present on all four CECs. The presence of phage EN34 on one of the CECs but not the others was interesting as it was a possibility that EN34 was near the breakpoint.

A screen for cosmids from the LL22NC03 chromosome 22-specific library was carried out by A. Johnson to isolate cosmids extending in both directions from the phage clone EN34. Two EN34-positive cosmids were selected and used in FISH analysis. The extent of overlap of these two cosmids was done by H. McDermid by hybridizing with probes along the phage and comparing restriction maps. One of these cosmids (N110A8), had the same results as the original phage clone, hybridizing only to the CEC of CM03. However, the other cosmid (N94C3) was present on the CECs of all four patients. This was not taken however as evidence that the breakpoint was contained within these clones because of the presence of repetitive sequences proximal to EN34. These repetitive sequences are also located at several more proximal locations in 22q11.2. If cosmid N94C3 contained some of the repetitive sequence, this would explain its hybridization to the CECs of all four type II patients. Thus at present, I believe it is unlikely that the distal breakpoints of the type II CECs are contained within these clones.

To determine how far beyond EN34 the second breakpoint of patient CM03 was located, probes distal to EN34 were tested by FISH. A cosmid representing locus D22S112 (cosmid address provided by M. Budarf, Children's Hospital of Philadelphia) was not present on the CEC (Figure 19d). Thus the second breakpoint of the CEC of CM03 is just distal to those of CM01, CM02, and CM20.

The location of the second breakpoint involved in the formation of the type IIb CEC of CM01 was still unknown after FISH analysis. Subsequent to this analysis, H. McDermid performed quantitative dosage analysis using a unique fragment isolated from plasmid CRKL. Results showed four copies of the CRKL locus, indicating symmetrical breakpoints of this type IIb CEC.

Figure 18. Results of FISH analysis of the four type II CECs of patients CM01, CM02, CM03, and CM20. The map is not to scale and was constructed based on information from Collins et al (1995), Frippiat et al (1995), Budarf et al (1996), and Budarf, personal communication. Provided above the line are relative locations of the loci and their somatic cell hybrid bin locations. Included for orientation purposes, are the locations of the CES critical region (CESCR) and the immunoglobulin (Ig) locus. Below the line are the clones used for FISH analysis and the results for each of the four individuals with a type II CEC. A (+) sign indicates the presence of the probe on the CEC and a (-) sign indicates its absence. Not all probes were tested on all four individuals. Numerous other probes present in somatic cell hybrid bin 8.1 were tested but not included in the table.



FISH Results
+/-

CM01	+	ND	ND	+	+	-	-	ND	-	-	-
CM02	+	+	+	+	+	-	ND	ND	-	-	-
CM03	+	ND	ND	+	+	+	-	-	-	-	ND
CM20	ND	ND	ND	+	+	-	ND	ND	ND	-	ND

ND = not done

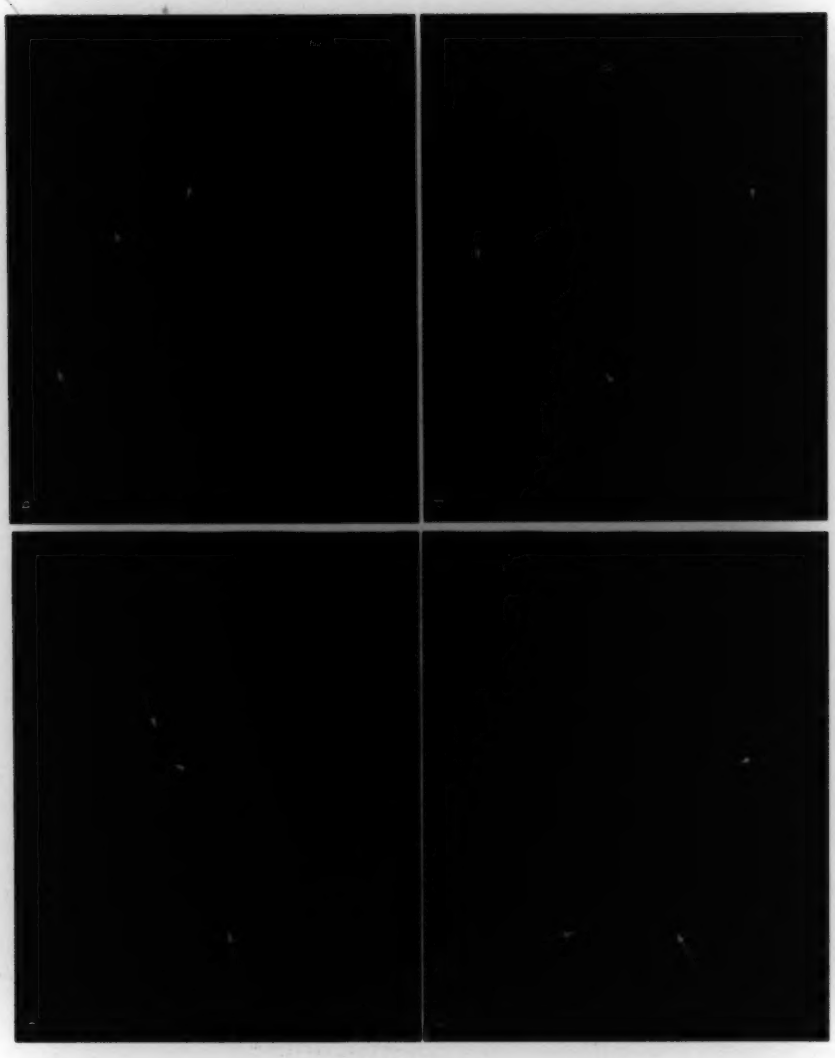
Figure 19. FISH analysis of type II CECs. The arrowheads denote the CEC and the arrows identify the normal chromosomes 22.

(a) Metaphase spreads of patient CM03 probed with plasmid CRKL, demonstrating hybridization to the CEC. Hybridization to the CEC was observed for CM01, CM02, and CM20 as well.

(b) CM02 probed with cosmid N110A8 representing locus EN34 demonstrating the absence of hybridization to the CEC. A lack of hybridization to the CEC was observed for CM01 and CM20 as well (not shown).

(c) Phage EN34 hybridized to the CEC of CM03. This is the first locus that differentiates the CEC of this patient from the others.

(d) Cosmid N19A11 representing locus D22S112 hybridized to the normal chromosomes 22 of CM03 but not the CEC, thus placing the CEC breakpoint in this patient just distal those of CM01, CM02, and CM20.



SUPERNUMERARY RING CHROMOSOME

Ohashi et al., in an abstract in 1993, reported the second case of a supernumerary ring chromosome 22 associated with CES. We have designated this patient CM16. The clinical features of CM16 at 4.5 years of age are fairly mild and are listed below.

- encephalocele (gap in skull with protruding brain material)
- hypertelorism (widely set eyes)
- low nasal bridge
- right preauricular pits and tags
- mild mental retardation

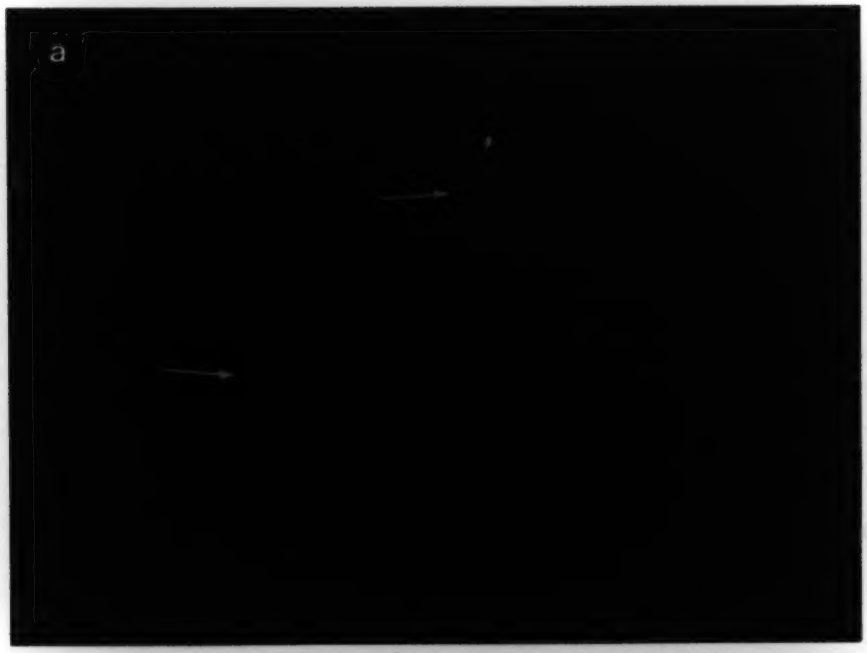
Cytogenetics performed by Ohashi and his group demonstrated the presence of the supernumerary ring chromosome in 86% of the metaphases examined from a peripheral blood sample. FISH analysis with a probe specific for all telomeres (ONCOR) revealed the absence of a telomere on this chromosome. A lymphoblastoid cell line was produced by Dr. Fukushima which was used in all subsequent studies. Preliminary FISH results of Mears (1995) demonstrated the presence of loci D22S9 and D22S181 on the ring, indicating that it was larger than the previous familial ring which included locus D22S57 but not ATP6E (Figure 3 on page 21).

Mears speculated that the precursor of the ring chromosome was an unstable CEC. Once the location of the distal type II CEC breakpoints was found, it became possible to test this hypothesis. I therefore extended this analysis, proceeding distally through band 22q11.2 to determine the size of the ring. Figure 20 shows FISH photographs of metaphase spreads of CM16 hybridized with a probe present on the supernumerary ring and one that is absent. The two normal chromosome 22s act as positive controls. Figure 21 lists the probes and cosmids used for this FISH analysis and their results. The duplication present in the supernumerary ring of CM16 is larger than all of the CECs characterized thus far. Thus, either this ring chromosome did not form by the breakage of a CEC, or there exists a larger CEC not yet characterized.

A limitation of FISH analysis in this case was the inability to distinguish between a single and a double ring (3 versus 4 copies of proximal 22q). Quantitative dosage analysis would be required in order to make this distinction, however this type of analysis requires the presence of the ring in almost all of the cells. The 86% mosaicism level in the peripheral blood sample may have been high enough to perform densitometric analysis. Since our analysis utilized DNA from the established lymphoblastoid cell line, we had to first determine the level of mosaicism in these cells, as the percentage of cells containing the ring chromosome may not be representative of that observed in the peripheral blood sample. Mears (1995) estimated that 50% to 60% of the cells possessed the ring chromosome.

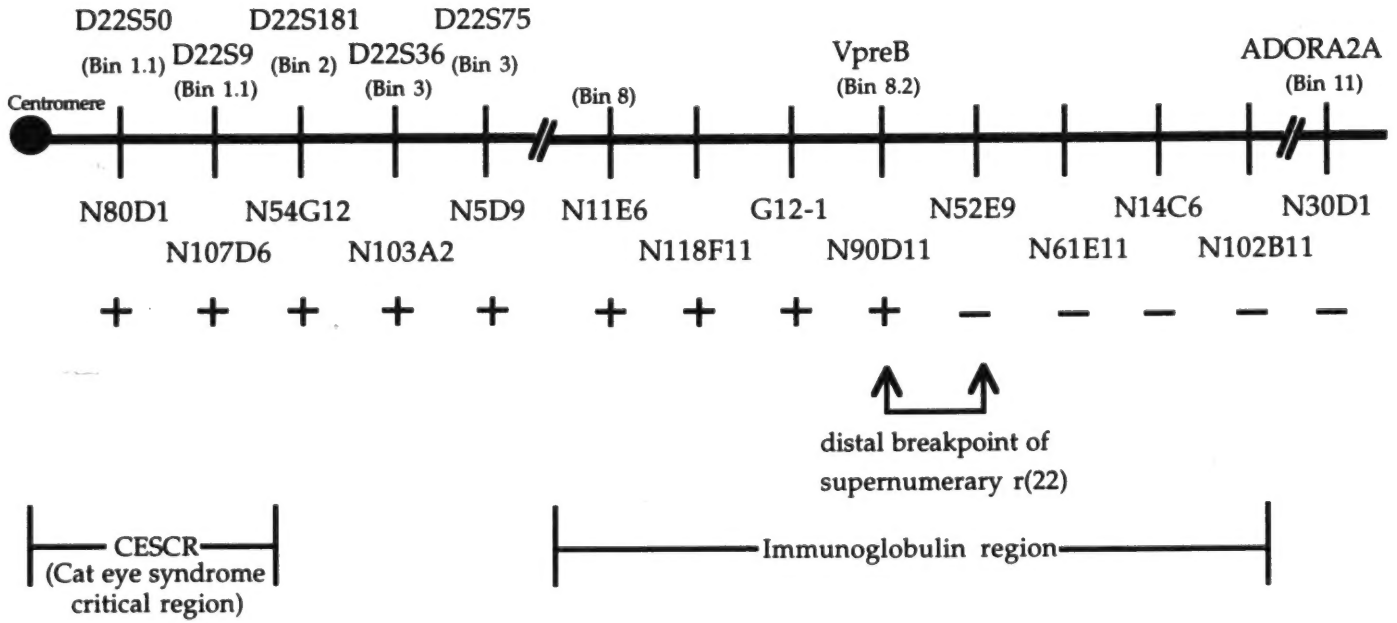
Random metaphase spreads from two of the slides used in FISH analysis were examined. All of the FISH analysis was performed using the same cell preparation. A total of 50 metaphase spreads were photographed and chromosome counts performed. Only those spreads without "stray" chromosomes were chosen for analysis. "Stray" chromosomes is a term I use referring to single chromosomes or a small group of chromosomes close to a metaphase spread such that it is uncertain whether the chromosomes are from the same cell. Stray chromosomes from neighboring cells could often be differentiated due to different condensation levels, however this was not always possible. Because the presence of these "strays" can lead to incorrect total chromosome counts, these spreads were avoided. Of 50 spreads photographed, 44 were included in the mosaicism calculation. The other 6 had ambiguous chromosome counts due to overlapping chromosomes and were thus excluded. 52% (23/44) of the spreads had a total of 47 chromosomes, with the extra one being the r(22). An additional 108 spreads were scored only for the presence or absence of the ring chromosome (no total chromosome count was done). Of these 108 spreads, 53 (49%) contained a ring chromosome and 55 (51%) did not. The level of 49% to 52% mosaicism in the cell line is thus considerably lower than that in the peripheral blood sample (in agreement with Mears 1995) and precluded dosage analysis.

Figure 20. Metaphase spreads of CM16 hybridized with two test cosmids flanking the q-arm breakpoint of the supernumerary r(22). Both cosmids are located within the immunoglobulin region. (a) Cosmid N90D11 representing locus VpreB from somatic cell hybrid bin 8.2. This cosmid is present on the r(22) (arrowhead), as well as the two normal chromosomes 22 (arrows). (b) Cosmid N61E11 is absent from the r(22) but present on the two normal chromosomes 22.


a

b

Figure 21. Results of FISH analysis of the supernumerary ring chromosome of patient CM16. A diagrammatic representation of proximal 22q is shown with the relative locations of loci and clones used in the analysis based on Collins et al (1995) and Kawasaki et al (1995). This map is not drawn to scale. Non-relevant portions of the map between somatic cell hybrid bins 3 and 8, and 8.2 and 11 have been excluded from the map and are marked by the double diagonal lines. Above the line are loci and in parentheses are the somatic cell hybrid bin locations according to Budarf et al (1996) or Budarf, unpublished results. Below the line are addresses selected from the chromosome 22-specific library, LL22NC03 (de Jong et al., 1989). The one exception is phage G12-1 from McDermid and Emanuel (1994). Refer to Table 9 for the references of these loci and clones used as FISH probes. The (+) or (-) sign below the cosmid designations indicates the presence or absence of hybridization, respectively, on the supernumerary ring chromosome. The interval containing the distal breakpoint of the r(22) chromosome is marked below the map.


D22S50
(Bin 1.1)
D22S9
(Bin 1.1)
D22S181
(Bin 2)
D22S36
(Bin 3)
D22S75
(Bin 3)
Centromere
(Bin 8)
VpreB
(Bin 8.2)
ADORA2A
(Bin 11)
N80D1
N107D6
N54G12
N103A2
N5D9
N11E6
N118F11
G12-1
N90D11
N52E9
N61E11
N14C6
N102B11
N30D1
+ + + + + + + + + − − − − −
distal breakpoint of
supernumerary r(22)
CESCR
(Cat eye syndrome
critical region)
Immunoglobulin region

DISCUSSION

Physical Mapping

The capacity of YACs to accommodate large inserts of human sequence has greatly facilitated physical mapping. The average insert size in the CEPH YAC library is 1.0 Mb (Chumakov et al., 1995). The sizes of the YACs utilized in our contig of the CES region ranged from 120 kb to 1490 kb. Two disadvantages of YACs are chimerism and instability. To determine chimerism in our study, we hybridized total yeast DNA containing YACs to human metaphase chromosome spreads. The results are listed in Table 10 on page 70. A drawback of this type of FISH analysis is that small chimeric regions may remain undetected. The alternative *in situ* method using Alu-PCR generated fragments as probes is even more likely to miss regions of chimerism. End cloning of the YAC insert is a more accurate method and will detect these small regions of chimerism. An additional advantage of this method is that the resulting clones can be used for end walking and as probes. It is however, more time consuming and difficult to perform than the FISH analysis. End cloning was used by C. Bell to confirm our FISH results for YAC 734B10. This YAC contains a small region of chromosome 21 that was originally missed by FISH analysis because of its weak signal. Closer examination revealed additional hybridization to a small chromosome believed to be chromosome 21 based on size and centromere location. The end clones later constructed by C. Bell confirmed this. I therefore feel that our method for detection of chimerism was accurate enough for our purposes and was an easier, less time consuming alternative to end cloning. I believe that the in situ hybridization of the YAC to metaphase chromosomes was sensitive enough to detect any major regions of chimerism which would have posed difficulties in the construction of the YAC contig and the placement of the YAC on a genomic PFGE restriction map.

The size of the YAC clones was determined by PFGE analysis and the results indicated that both the same isolate and different isolates of the

same strain could contain YACs of different sizes. A single isolate of both 745G7 and 925G12 each contained four YACs of different sizes. Hybridization analysis revealed that each of the four 745G7 YACs contained locus D22Z2 and each of the four 925G12 YACs contained locus D22S9. This indicates that these YACs represent the same original YAC but with differing deletions. In contrast, each isolate of the yeast strain containing YAC 803G9 contained only one YAC with a size of either 200kb or 580kb. Both sizes hybridized to D22S9 and thus are also most likely derived from the same parent YAC by deletion. Analysis of more isolates found a total of 12 different sizes of this YAC, indicating instability (McDermid et al., 1996).

FISH analysis with the 15 YACs listed in Table 10 on page 70 showed that 11 of them, or 73%, were chimeric, hybridizing to more than just chromosome 22 and the CEC. An alternative explanation for the *in situ* hybridization of YACs to multiple chromosomes is that such YACs are not chimeric but are cross-hybridizing to repeats or related regions. The close proximity of our region of focus to the centromere makes this possibility especially plausible as centromeric and pericentromeric regions are known to contain sequence such as α-satellite repeats that cross hybridize with the sequence of other chromosomes. For example, I found that a cosmid from the map of Collins et al (1995) placed near the centromere of chromosome 22, hybridizes to chromosome 22 as well as the pericentromeric region of a D-group chromosome (Figure 11b on page 75). This D-group chromosome is likely chromosome 14 because of the similarities shared between the centromeric and pericentromeric regions of chromosomes 14 and 22 (Choo et al., 1990; Hulsebos et al., 1996).

Two pieces of evidence support our conclusion of chimerism. First, the chimerism of one YAC was confirmed by a second method. FISH analysis of YAC 734B10 on the metaphase spreads of CM02 demonstrated hybridization to the CEC, chromosome 22, and chromosome 21. Chimerism was confirmed by C. Bell, who cloned the ends of the YAC and found that the proximal end clone hybridized to chromosome 21 and the distal end clone to chromosome 22, using a monochromosomal hybrid panel (H. McDermid & A. Johnson). The other piece of convincing

evidence was that the PFGE map of the chimeric YACs had regions that did not match to the genomic map, whereas the non-chimeric YACs matched over the their entire lengths (McDermid et al., 1996). The YACs were placed on the PFGE map by restriction fragment mapping using two enzymes (NotI and AscI) with an 8 base pair recognition sequence. They were also tested by Southern hybridization for the presence of the probes placed on the PFGE map. Those YACs shown by FISH analysis to be chimeric had fragments generated by the restriction enzymes that were not present on the genomic map. Moreover, based on the probes present, some YACs spanned a distance on the map which was considerably smaller than their actual size as determined by PFGE. Chimerism, rather than the hybridization to repetitive sequence, would explain these discrepancies.

In summary, *in situ* hybridization of the YACs was an effective method of determining chimerism and helped in the construction of the YAC contig of the proximal portion of 22q11.2 (McDermid et al., 1996). However, due to the high rate of chimerism of the YACs in this region, a BAC/PAC contig was subsequently constructed. This new contig will be very useful in the further characterization of the region because of the greater insert stability in the BACs and PACs. These contigs and physical maps are a prerequisite to the search for genes involved in CES.

Cat Eye Chromosomes

The CECs are divided into two groups based on the extent of duplication. The type I CECs are smaller, with symmetrical breakpoints occurring distal to the CESCR, resulting in four copies of this region. The larger type II CECs are duplicated for the CATCH22 deletion region and are divided into two subgroups. Figure 12 on page 78, depicts ideograms of a normal chromosome 22, a type I CEC, and type IIa and type IIb CECs. Both subgroups possess four copies of the CESCR. The type IIa CECs are asymmetrical with only one extra copy of the CATCH22 region, whereas the type IIb CEC of CM01 is symmetrical with two extra copies of the deletion region. The four type II individuals studied in this project do not

appear to be more severely affected than those without duplication of the CATCH22 region. In particular, CM01 (four copies) lacks ocular coloboma and a congenital heart defect, two of the cardinal features of CES (Rosenfeld et al., 1984). The apparent lack of any additional features in the type II individuals suggests that duplication of the VCFS/DGS deletion region has no or little phenotypic effect. This may partly explain why no individuals have been found with a duplication of only this region. These individuals likely exist as reciprocal products of the mechanism resulting in the CATCH22 microdeletion (see below), however would be difficult to detect without performing dosage analysis.

- Breakpoints of type I CECs

From Mears et al (1994), Mears (1995), H. McDermid (unpublished results), and from my analysis there are nine confirmed type I CECs (CM04, CM05, CM06, CM07/CM08, CM09, CM10, CM11, CM12, and CM17). Both breakpoints involved in the formation of each of these CECs with the exception of one, have been demonstrated by FISH, dosage, or RFLP analysis to be within the 1Mb D22S181-D22S36 interval (CM17 was the only new patient since Mears et al., 1994 and Mears 1995). In all of these individuals with the exception of CM04 and CM12, both CEC breakpoints have been further localized to a 450-650kb D22S427-D22S36 interval by FISH (this thesis) and dosage analysis (this thesis; McDermid unpublished). DNA from individual CM12 was not available for further study, and CM04 was polymorphic for the probe used in the quantitative dosage analysis.

Patient CM11 has an interstitial duplication in a "normal" chromosome 22 in addition to a type I CEC (Mears et al., 1994). The interstitial duplication is also present in the father (YM11) (Mears 1995). Quantitative dosage analysis performed by Mears et al (1994), Mears (1995), and H. McDermid (unpublished results) indicate that the interstitial duplication encompasses only locus D22S36. Figure 22 depicts these results which suggest placement of both CEC breakpoints in the D22S427-D22S36 interval for CM11. Another patient, CM04, also has an interstitial duplication in addition to a CEC. The mother of this patient is known to

possess a supernumerary CEC however was unavailable for study. Therefore, I could definitively place only one breakpoint of the CEC of this individual by FISH analysis.

In summary, of the 16 breakpoints of the 8 confirmed type I CECs examined in this study, 15 are located in the D22S427-D22S36 interval. The only remaining breakpoint is that of patient CM04, which at present cannot be located with certainty.

Figure 22. Duplications present in CES patient CM11 and his father, YM11. The shaded area indicates the location of the interstitial duplication (Mears et al., 1994; Mears 1995) and the arrows indicate the location of the CEC breakpoints of CM11 (McTaggart, this thesis; McDermid, unpublished results). Breakpoint localization of the type I CEC of CM11 was possible because of the information provided by dosage analysis of YM11 using D22S427 (McDermid, unpublished results).

Individual / Locus	YM11	CM11
D22S181	2	4
D22S427	2	4
D22S36	3	3
D22S75	2	2

• Breakpoints of type II CECs

Preliminary analysis by Mears et al (1994) demonstrated by dosage analysis that these CECs had more extensive duplications than the type I CECs and that two of the three examined were asymmetric. I have designated these asymmetric CECs as type IIa and since this analysis, have characterized one more. The symmetrical CEC of Mears et al (1994) has been classified as type IIb.

All three proximal breakpoints of the type IIa CECs have been localized to the D22S427-D22S36 interval using FISH (this thesis) and dosage analysis (this thesis; McDermid unpublished). The distal breakpoints of these three CECs and that of CM01 were localized by my FISH analysis. FISH results placed two of three type IIa breakpoints as well as one of the two breakpoints of CM01 distal to CRKL but proximal to EN34. The remaining type IIa breakpoint was distal to EN34 but proximal to D22S112, the next distal probe tested. Thus, the breakpoints appeared to be clustering somewhere in the vicinity of EN34 although, at least one is slightly different from the rest. The second CEC breakpoint of CM01 was placed in the CRKL-EN34 interval as a result of dosage analysis (H. McDermid, unpublished results).

To expand the EN34 region, two cosmids were isolated using a fragment of phage EN34, and tested by FISH on these four patients. Cosmid N110A8 demonstrated the same results as phage EN34 (absent on three of four CECs), however, cosmid N94C3 was present on all four CECs. Two possibilities thus exist to explain this finding: (1) the distal breakpoints of CM01, CM02, and CM20 are within the EN34 cosmid contig, or (2) the cosmid N94C3 contains low copy repetitive sequences also found in a more proximal location on 22q. Further characterization of cosmids N110A8 and N94C3 is necessary before any conclusions can be made. To test the possibility of the cross-hybridization of putative repeats contained within cosmid N94C3 at a more proximal location, this cosmid could be hybridized to metaphase spreads of an individual with a type I CEC. The presence of N94C3 on the type I CEC would confirm the presence of repetitive sequence in this cosmid. Its absence would only suggest the

absence of repeats from the centromere to the location of the CEC breakpoint between D22S427 and D22S36. It would not exclude the possibility of the presence of repeats distal to this. FISH analysis was performed by D. Shkolny with cosmid N94C3 on CM07. Fluorescent signal was not consistently evident on the CEC in all metaphases examined and thus no conclusions could be drawn. This experiment should be repeated to determine if this inconsistency exists on all CECs or if it was a result of the one hybridization that was performed.

An alternative explanation somewhat related to the idea of the existence of low copy repetitive sequences is the suggestion of the presence of a large duplicated segment in this region of chromosome 22 (Budarf, unpublished results). Preliminary evidence indicates that this region appears to have been recently duplicated and the two copies are very similar in sequence. In addition to this unpublished information, there is evidence that the recurrent 11;22 translocation occurs in this region as well (Barnoski et al., 1995) but cannot be localized with certainty since all of the clones in the region hybridize to both sides of the translocation breakpoint. This would not be unexpected if the breakpoint was within a tandem duplication region. This putative duplication is presently being characterized and exists between the type I and type II CEC breakpoint regions.

Historically, it has always been of interest that no der(22)t(11;22)(q23;q11) cases exist with coloboma (except for the one case of Simi et al., 1992). The der(22) was originally believed to possess more chromosome 22 material than the CECs and thus the absence of coloboma was believed to be due to either the presence of some inhibitory factor present on the additional portion of 22q or to the presence of an inhibitory factor on the duplicated portion of chromosome 11. I have shown that the type II CEC duplications extend further distally than the der(22) and that one of these individuals (CM03) has coloboma. This lends support to the argument that interference with the production of coloboma in the der(22)t(11;22)(q23;q11) syndrome is a result of the duplicated portion of chromosome 11.

- CEC Breakpoint Summary: Involvement of Repetitive Sequences

According to the maps of Collins et al (1995; 1997), numerous low copy repeats exist in proximal 22q. Interestingly, the locations of these repeats flank the regions in which the CEC duplication breakpoints occur. Figure 23 depicts the locations of low copy repeats in proximal 22q (Collins et al., 1995; Collins et al., 1997) in relation to the CES duplication breakpoints.

Interestingly, the CEC duplication breakpoint regions correspond to the CATCH22 deletion breakpoint intervals (shown in Figure 24). The type I CEC interval corresponds to the proximal CATCH22 deletion breakpoint interval and the type II CEC interval to the more common distal deletion breakpoint interval of CATCH22. This lends further support to the notion that 22q11.2 possesses regions of instability, leading to frequent rearrangements. As depicted in Figure 23 on page 115, these regions contain low copy repetitive sequences such as GGT, D22S131, D22S287E, D22S207, and 22.71 which may facilitate misalignment and unequal crossing over. GGT is the name of a gene encoding gamma glutamyl transferase and D22S287E, D22S131, and D22S207 represent what were once believed to be unique sequences.

Figure 23. Low copy repeats in 22q11.2. This map was constructed from information provided in Collins et al (1995 & 1997). In the case of a discrepancy, the most recently published data was used. Repeat sequences are depicted below the map, and the duplication breakpoint intervals of the CECs, the deletion breakpoint intervals of CATCH22 (Figure 4), and the 22q breakpoint of the supernumerary r(22) of patient CM16 are above the map. The map is not drawn to scale and the order of the repeats in each interval are not necessarily in a proximal to distal order. Loci on the map beginning with "S", should be prefaced with "D22". GGT refers to the gamma glutamyl transferase gene.

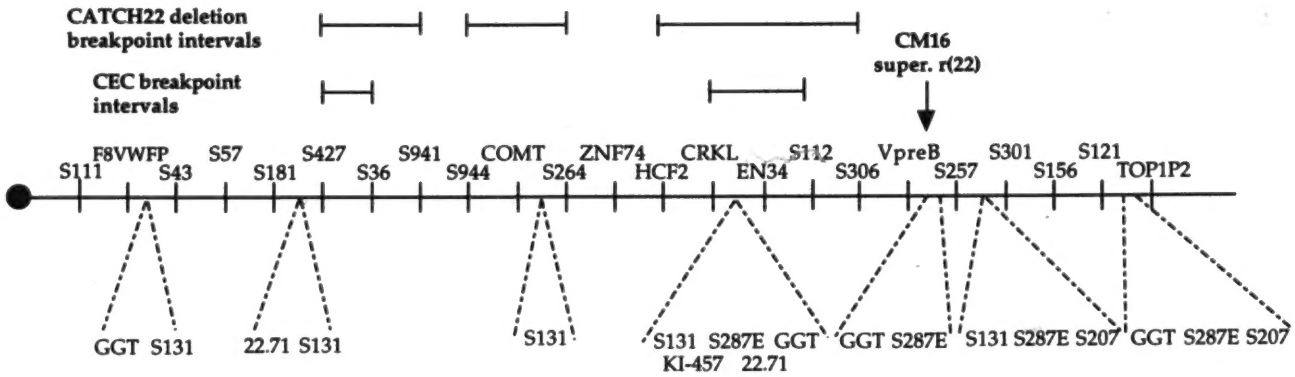
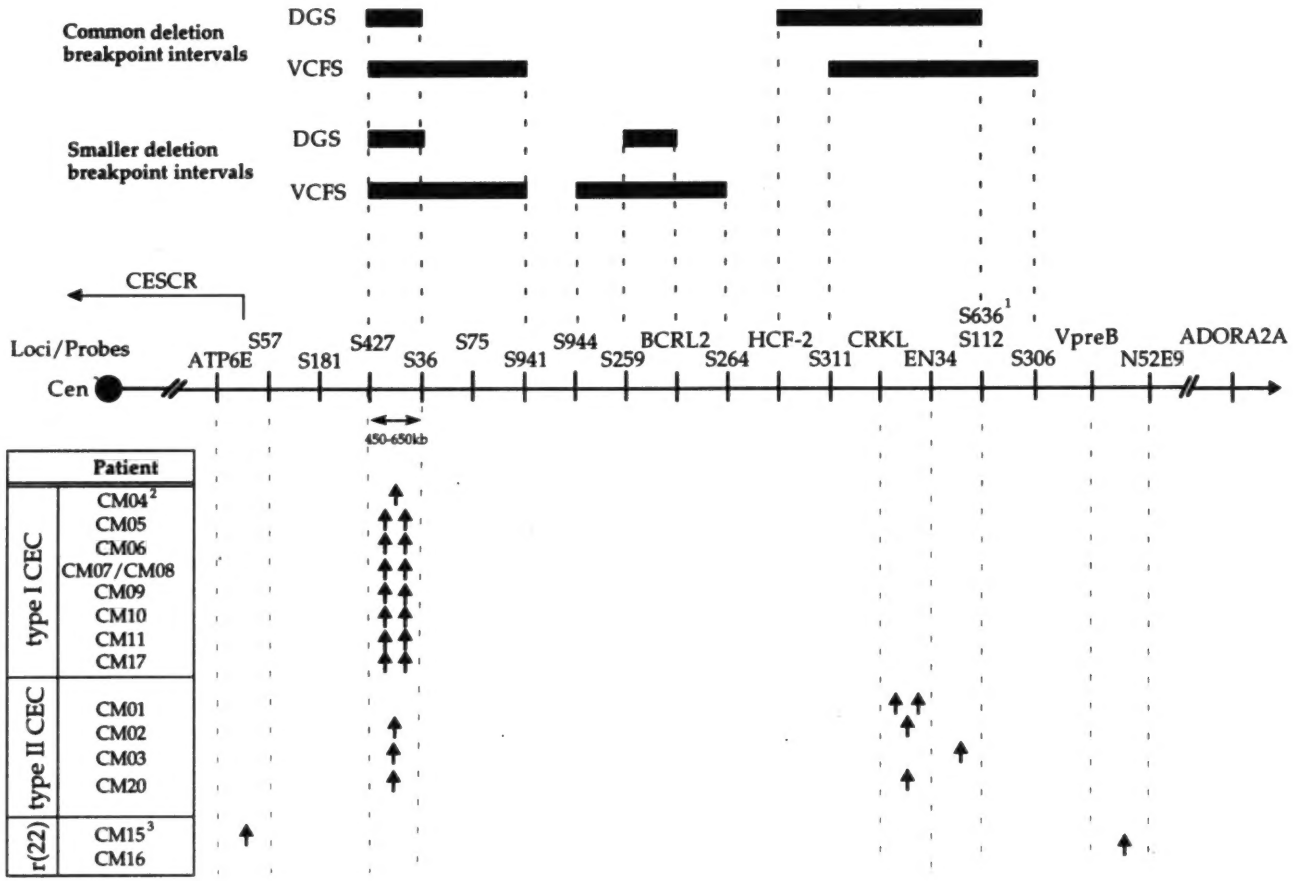


Figure 24. Breakpoint summary of CES duplications and CATCH22 (DGS and VCFS) deletions. Deletion breakpoint intervals (solid boxes) in DGS and VCFS have been determined using different probes (Morrow et al., 1995; Carlson et al., 1997; Driscoll et al., 1995; Li et al., 1995; Driscoll & Emanuel, 1996) and thus are depicted separately. The duplication breakpoints of the type I and type II CECs as well as the r(22)s are represented by arrows. Each individual has two breakpoints. The relative order of loci on the map has been summarized from Budarf et al (1996), Collins et al (1995), Frippiat et al (1995), and Kawasaki et al (1995). Loci beginning with an "S" should be preceeded by "D22". The map has not been drawn to scale.

[1] The order of these loci cannot be determined. Collins et al (1995) places D22S636 proximal to D22S112 and Budarf et al (1996) places them in the reverse order.

[2] Only one breakpoint could be determined for this individual because of a polymorphism for the probe representing the D22S427 locus.

[3] The 22q breakpoint of the supernumerary r(22) from Mears et al (1995) is included because of its importance in delineation of the distal boundary of the CESCR and for comparison to the supernumerary r(22) of patient CM16.


Common deletion breakpoint intervals
DGS
VCFS
Smaller deletion breakpoint intervals
DGS
VCFS
CESCR
Loci/Probes
Cen
ATP6E
S57
S181
S427
S36
S75
S941
S944
S259
BCRL2
S264
HCF-2
S311
CRKL
EN34
S636[1]
S112
S306
VpreB
N52E9
ADORA2A
450-650kb
Patient
type I CEC
CM04[2]
CM05
CM06
CM07/CM08
CM09
CM10
CM11
CM17
type II CEC
CM01
CM02
CM03
CM20
r(22)
CM15[3]
CM16

• Repetitive Sequence Involvement in Other Conditions

There are numerous other conditions resulting from rearrangements believed to be facilitated by repetitive sequence. The most striking of these are Charcot-Marie-Tooth disease type 1A (CMT1A) and hereditary neuropathy with liability to pressure palsies (HNPP). CMT1A is a duplication of and HNPP is a deletion of the same 1.5Mb region on 17p11.2 (Lupski et al., 1992; Pentao et al., 1992; Chance et al., 1993; Mariman et al., 1993; LeGuern et al., 1994) and are thus believed to be reciprocal products of an unequal crossover event. CMT1A is an autosomal dominant, completely penetrant form of demyelinating neuropathy. The average age of onset is 12 years of age, and the diagnostic feature is slowed nerve conduction velocity (OMIM#118220). Other reported features are neonatal hypotonia, muscle weakness and atrophy, pes cavus (very high arch of the foot), and absent deep tendon reflexes. HNPP is also an autosomal dominant neuropathy with onset usually in adolescence but is less severe than CMT1A. Characteristic of HNPP are localized pressure palsies following local minor trauma to peripheral nerves. HNPP usually manifests as numbness, muscle weakness and atrophy. Nerve conduction velocities may be mildly affected but not to the degree as in CMT1A. Other features include brachial neuritis (pain in the shoulder followed by muscle weakness and wasting), scoliosis, deafness, hypotelorism, and pes cavus (OMIM#162500). Contained within the duplicated/deleted region is the PMP22 gene, believed to be dosage sensitive (Patel et al., 1992; Takahashi et al., 1992; Timmerman et al., 1992; Matsunami et al., 1992; Valentijn et al., 1992). PMP22 is expressed in Schwann cells (Patel et al., 1992), the cells of the peripheral nervous system that form a sheath around the nerves.

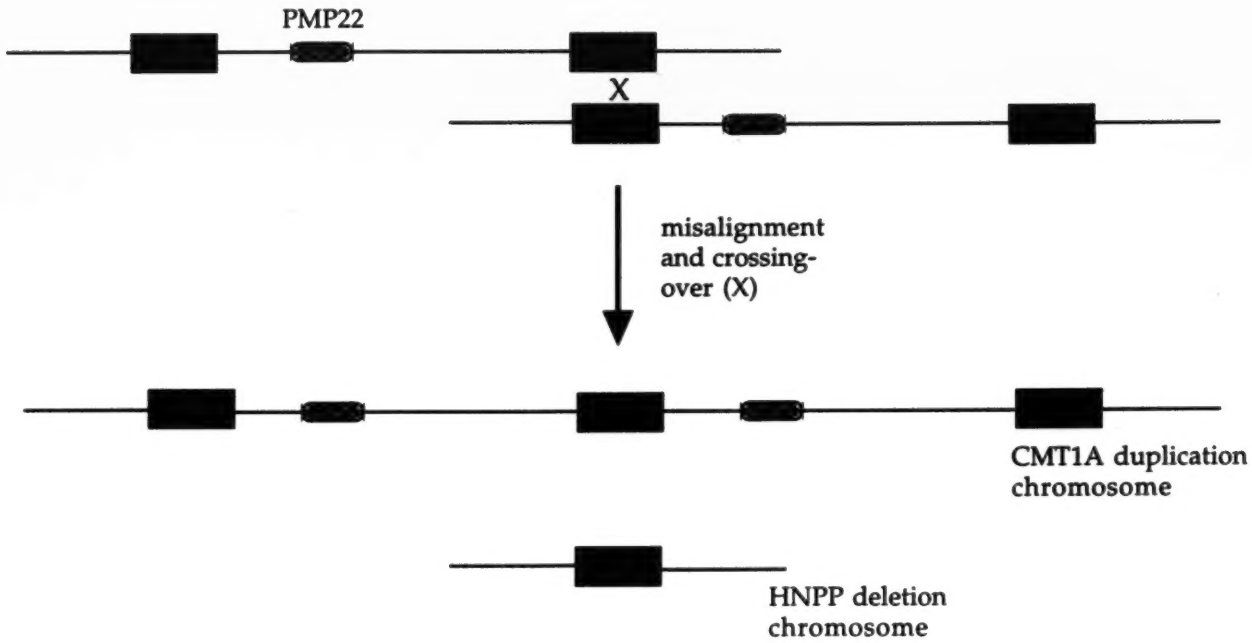
Characterization of the region involved in the duplication/deletion revealed the presence of low copy repeats flanking both sides of the 1.5Mb duplicated segment (Pentao et al., 1992). Each of these repeats is called a REP unit, and is approximately 30kb in length (Reiter et al., 1996). CMT1A and HNPP individuals possess three copies and only one copy of this CMT1A-REP unit, respectively. The REP unit on an HNPP deleted chromosome and the middle repeat on a CMT1A duplicated chromosome are "hybrids". This indicates that misalignment likely facilitated by these

repeated sequences themselves and a subsequent crossover event resulted in the generation of the duplication/deletion products. The junctions of these hybrid repeats were characterized, revealing "hotspots" of recombination. Reiter et al (1996) identified a 1.7kb "hotspot" in 75% of CMT1A patients and 84% of HNPP patients. The concordance of HNPP and CMT1A breakpoints demonstrated these conditions were reciprocal products of the same rearrangement. Thus the CMT1A duplication and HNPP deletion are the result of misalignment of the two REP units flanking the 1.5Mb region containing the PMP22 gene, and recombination at a "hotspot" within the REP unit. The generation of the duplication and deletion is depicted in Figure 25. Interestingly, a "mariner" transposon-like element (MITE) is located near the "hotspot", possibly indicating strand exchange via the action of a transposase (Reiter et al., 1996).

There are several more examples of conditions resulting from rearrangements facilitated by repetitive sequence. One of them is the deletion of the STS gene responsible for X-linked ichthyosis. X-linked ichthyosis is characterized by a scaling of the skin, especially on the scalp, ears, neck, trunk, and flexures (knees, elbows, etc). Corneal opacities are also reported (OMIM#308100). The scales are a result of the accumulation of undegraded cholesterol sulfate (Elias et al., 1984) which is a result of a deficiency of 3-B-hydroxysteroid sulfatase. An estimated 85-90% of cases are due to the deletion of the entire STS gene (Yen et al., 1987; Gillard et al., 1987; Ballabio et al., 1987; Shapiro et al., 1987; Bonifas et al., 1987; Yen et al., 1990). Knowlton et al (1989) isolated S232 sequences that flanked the STS gene. The breakpoints involved in the deletion were within these sequences in 24 of 26 individuals studied. Characterization of the S232 elements, revealed the similarity to VNTR sequences (Li et al., 1992). It has been suggested that VNTR sequences may stimulate homologous recombination (Wahls et al., 1990).

Another case of repetitive sequence facilitating a chromosomal rearrangement is demonstrated by hemophilia A. Most cases of hemophilia A result from different mutations however, 45% of cases result from an inversion. This inversion is the result of an

Figure 25. Generation of the reciprocal duplication/deletion products of CMT1A and HNPP. Misalignment facilitated by 30 kb CMT1A-REP units flanking the 1.5Mb region containing the candidate PMP22 gene followed by a crossover event, results in the production of hybrid REP units and a 1.5Mb duplication and deletion.



intrachromosomal recombination event between sequence present within the gene and sequence present upstream in the opposite orientation (Lakich et al., 1993). If the recombination event were interchromosomal, a dicentric and an acentric chromosome would be the result.

Generation of the recurrent CATCH22 deletions in 22q11.2 also likely involves the misalignment of repetitive sequences. Flanking the deletion breakpoint are characterized repetitive sequences (depicted in Figure 23- Collins et al., 1995 & 1997). The breakpoints involved in the formation of the CECs also occur in this region, and thus may involve these repetitive sequences as well. To elucidate the mechanisms of formation and involvement of repetitive sequences in the generation of inverted duplications, it may be instructive to look at an analogous region on a different chromosome.

- Inverted Duplication Chromosome 15/Inv dup(15)

A supernumerary small chromosome of unknown origin is called a "marker" chromosome. The reported incidence of these markers in prenatal diagnosis is from 0.4 to 1.5 per 1000 (Warburton et al., 1991; Brøndum-Nielsen & Mikkelson, 1995; Sachs et al., 1987; Blennow et al., 1994). The incidence in newborns ranges from 0.14 to 0.7 per 1000 (Hamerton et al., 1975; Gravholt & Friedrich 1995). The majority (87% to 94%) of those identified are phenotypically unaffected (Warburton et al., 1991; Gravholt & Friedrich 1995). Very few supernumerary markers are associated with a particular phenotype, or syndrome. One of these is the CEC, responsible for CES. Another is the inverted duplication chromosome 15 [inv dup(15)], most of which have a normal phenotype.

The CEC shows a great number of similarities to the inv dup(15). The inv dup(15)s are the most common supernumerary chromosome, accounting for 40-50% of all supernumerary structurally abnormal chromosomes (Schreck et al., 1977; Mattei et al., 1984; Buckton et al., 1985; Blennow et al., 1994). Their structure is very similar to the CEC: dicentric, bisatellited, involved in satellite associations, and possessing two centromeric regions but only one primary constriction, indicating stability

through either centromere inactivation or co-operation (Schmid et al., 1986; Fujita et al., 1980; Knight et al., 1984; Maraschio et al., 1981; Wisniewski et al., 1979; Stetten et al., 1981; Schreck et al., 1977; Luke et al., 1994). They are composed of duplications of 15p and the proximal portions of 15q and their formation, like the CECs, involve two breakpoints.

Proximal 15q, like proximal 22q, is involved in other rearrangements as well. The Prader-Willi syndrome (PWS) and Angelman syndrome (AS) result from the deletion of paternal and maternal contributions respectively, of the proximal portion of 15q (Pembrey 1989; Knoll et al., 1989; Butler & Palmer 1983; Ledbetter et al., 1981). Unlike 22q, this region of 15q is imprinted, and is methylated differently depending on the parent of origin (Driscoll D.J. et al., 1992; Clayton-Smith et al., 1993; Dittrich et al., 1992). These methylation differences are thought to cause expression differences of genes in the PWS/AS region which would account for the different phenotypes of AS and PWS in individuals with a deletion of the same region (Glenn et al., 1993; Reed & Leff 1994; Woodage et al., 1994; Ning et al., 1996; Wevrick et al., 1994).

PWS is characterized by diminished fetal activity, neonatal hypotonia, mental retardation, short stature, small hands and feet, hypogonadism, hypopigmentation, reduced sensitivity to pain, an increased risk for leukemia, and obesity due to hyperphagia or overeating,. Typical facial features include almond shaped eyes, strabismus, narrow face, full cheeks, a thin upper lip and down-turned corners of the mouth (OMIM# 176270). The average age of PWS patients (25-30 years) is reduced due to cardiac failure and diabetes that result from the obesity. AS is characterized by mental and motor retardation, jerky, or ataxic movements, seizures, hypotonia, absence of speech, tongue thrusting, hypopigmentation, bouts of uncontrollable laughter, and a happy disposition. Facial features include a large mandible and widely spaced teeth (OMIM#105830).

The majority of these patients (greater than 50% of AS and from 70-80% of PWS) possess similar interstitial deletions of 15q11q13 (Pembrey et

al., 1989; Knoll et al., 1989; OMIM#176270). Evidence indicates that the proximal interstitial deletion breakpoints in PWS/AS patients cluster in two regions (Donlon et al., 1986; Knoll et al., 1990; Christian et al., 1995; Mignon et al., 1996). Unlike the CATCH22 syndrome phenotypes which appear to be a varying phenotypic spectrum of the same deletion, the two deletion 15q syndromes differ as a result of the effects of imprinting. Therefore, PWS and AS can also result from uniparental disomy (UPD). UPD occurs when both copies of a region in an individual are received from one parent (Engel 1980). Maternal UPD accounts for almost all of the cases of PWS that do not have an interstitial deletion (Nicholls et al., 1989; Robinson et al., 1991; Purvis-Smith et al., 1992; Cassidy et al., 1992; Robinson et al., 1993a). Paternal UPD accounts for some cases of AS but is less commonly reported than UPD in PWS (Robinson et al., 1993a; Malcolm et al., 1991; Nicholls et al., 1992; Mutirangura et al., 1993). It has been suggested that some cases of UPD are the result of correction of a trisomy 15 (Purvis-Smith et al., 1992; Cassidy et al., 1992). There are also cases reported in which PWS/AS phenotypes are associated with the presence of a supernumerary inv dup(15), however this is a result of uniparental disomy (UPD) for the two normal chromosome 15s rather than the presence of the supernumerary chromosome (Fujita et al., 1980; Wisniewski et al., 1980; Ledbetter et al., 1982; Robinson et al., 1993b; Mattei et al., 1984; Buckton et al., 1985; Maraschio et al., 1981).

The inv dup(15) chromosomes are divided into groups based on the extent of duplication. These classes demonstrate some correlation between genotype and phenotype (Leana-Cox et al., 1994; Cheng et al., 1994; Nicholls et al., 1989; Shibuya et al., 1991; Robinson et al., 1993). The larger class of markers contain the PWS/AS region and demonstrate a more severe phenotype than the smaller markers which are not duplicated for this region. Common features of individuals possessing the larger inv dup (15)s include developmental delay, varying degrees of mental retardation, hypotonia, seizures, autism, abnormal speech, and mild facial dysmorphia including strabismus, downslanting palpebral fissures, epicanthal folds, and low set posteriorly rotated ears (Schreck et al., 1977; Wisniewski et al., 1979; Maraschio et al., 1981; Schmid et al., 1986; Schinzel et al., 1990; Grammatico et al., 1994; Luke et al., 1994; Van Der Smagt et al., 1996; Fletjer

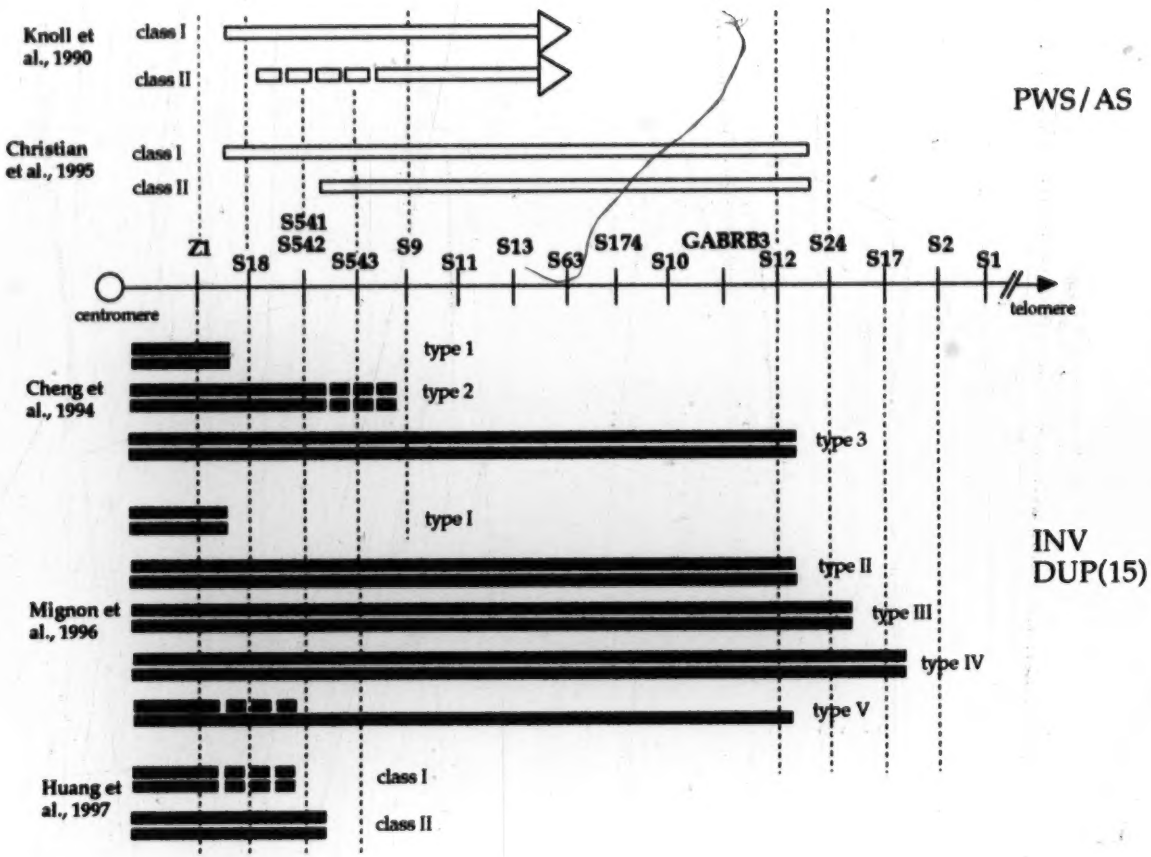
et al., 1996). The extent of duplication beyond the PWS/AS region does not seem to affect phenotype, thus it is the presence or absence of this region on the inv dup(15)s that is correlated with phenotype. Individuals possessing the smaller inv dup(15) are usually clinically normal but may manifest mild mental retardation and/or mild dysmorphic features (Stetton et al., 1981; Knight et al., 1984).

As with CATCH22 and the CEC, there is some evidence that the breakpoints involved in the formation of the inv dup(15)s are clustering (Cheng et al., 1994; Mignon et al., 1996; Huang et al., 1997). Figure 26 summarizes the results of studies characterizing the breakpoints. Also summarized in this figure are the locations of the breakpoints of the deletions associated with AS and PWS, which also appear to be clustering and correspond to the duplication breakpoints of the inv dup(15)s (Christian et al., 1995; Knoll et al., 1990).

Two classes of inv dup(15)s exist which have a normal phenotype and symmetrical breakpoints located in one of two intervals near the centromere (Cheng et al., 1994; Mignon et al., 1996; Huang et al., 1997). Both of these intervals correspond to the proximal deletion breakpoint intervals identified in PWS/AS (Knoll et al., 1990; Christian et al., 1995). The one distal deletion breakpoint interval identified in PWS/AS (Christian et al., 1995; Knoll et al., 1990) corresponds to the interval in which the breakpoints of the larger inv dup(15)s are located (Cheng et al., 1994; Mignon et al., 1996). These larger inv dup(15)s have the characteristic severe inv dup(15) phenotype as a result of the duplication of the PWS/AS region. Mignon et al (1996) identified three additional types of inv dup(15)s. One of these was an asymmetrical inv dup(15) with one breakpoint located in the interval closest to the centromere and the other located in the interval of the larger inv dup(15)s (Cheng et al., 1994). The other two were symmetrical with breakpoints located in intervals distal to the larger chromosomes of Cheng et al (1994). One of these intervals corresponds to the interval in which several interstitial duplication/ triplication breakpoints occur (Mutirangura et al., 1993; Holowinsky et al., 1993; Schinzel et al., 1994b).

The clustering of breakpoints in 15q, like 22q, is probably the result of the presence of repetitive sequences. Donlon et al (1986) provide evidence of repetitive sequences in this region. Thus, the numerous different types of rearrangements suggest genomic instability of the region as a result of repetitive sequences.

Figure 26. Breakpoint clustering in 15q. Summary of the duplication breakpoint intervals of the inv dup(15)s (below the map) and breakpoint intervals of PWS/AS deletions (above the map). The filled boxes indicate duplications of the inv dup(15)s. The discontinuous filled boxes indicate a breakpoint interval. The open boxes above the map represent deletions. The discontinuous boxes indicate a breakpoint interval. The arrows on the deletion boxes of Knoll et al (1990) indicate that D15S13 was the most distal locus tested. The map has not been drawn to scale and all loci beginning with an "S" should be preceeded by "D15". The relative order of loci has been summarized from Huang et al (1997), Cheng et al (1994), and Christian et al (1995).


Knoll et al., 1990
class I
class II
Christian et al., 1995
class I
class II
PWS/AS
Z1
S18
S541
S542
S543
S9
S11
S13
S63
S174
S10
GABRB3
S12
S24
S17
S2
S1
centromere
telomere
Cheng et al., 1994
type 1
type 2
type 3
Mignon et al., 1996
type I
type II
type III
type IV
type V
INV
DUP(15)
Huang et al., 1997
class I
class II

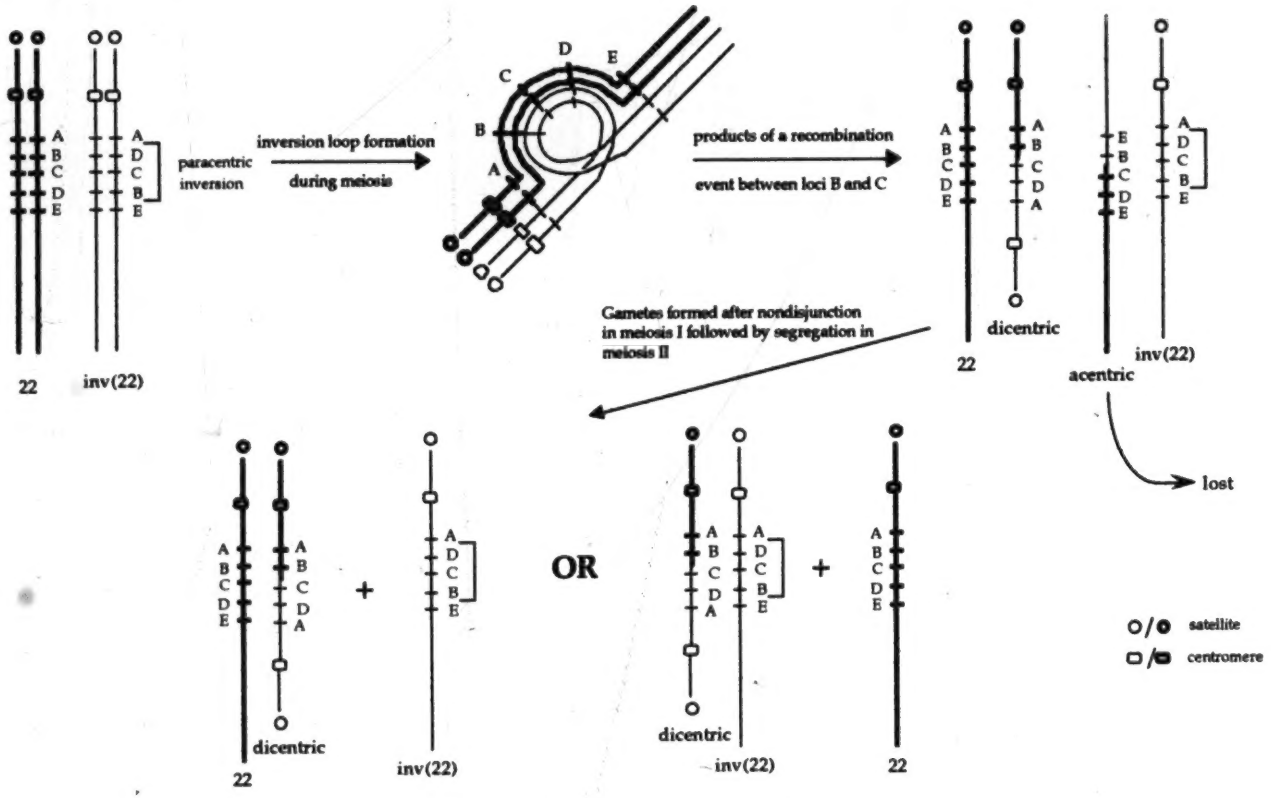
• Formation of Inverted Duplication Chromosomes

Several mechanisms have been postulated for the formation of inv dup (15)s (Schreck et al., 1977; Van Dyke et al., 1977) which can also apply to formation of the CECs due to the similarities in structure of these two chromosomes. Both mechanisms favored in the literature involve misalignment followed by a recombination event and then non-disjunction. A third model, the ARRC22 model, is a modification of the first two models, proposed by Mears (1995), to account for the rearrangements observed in 22q11.2.

The first mechanism requires a paracentric inversion in one of the parents (Schreck et al., 1977). Figure 27 depicts the events that would generate an inverted duplication chromosome. Heterozygosity for a paracentric inversion in one of the parents could lead to an inversion loop during pairing in meiosis I of gametogenesis. A recombination event within this loop would result in the generation of both an acentric and dicentric fragment. Subsequent nondisjunction in meiosis I and loss of the acentric fragment, followed by segregation in meiosis II, could result in a gamete containing an inverted duplication chromosome in addition to the normal complement.

There are few reported cases of paracentric inversions involving chromosomes 15 or 22 (Del Porto et al., 1984). The small size and mostly G-light appearance of chromosome 22 make the detection of paracentric inversions difficult. Part of the problem in determining if paracentric inversions in one of the parents was the precursor to these dicentric chromosomes is that paracentric inversions may not be detected cytogenetically. Examination of the parents of the cases reported by Mears et al (1994) failed to detect any chromosomal abnormalities, however, the resolution at which this analysis was done is unknown. An outcome of this type of event is the generation of an asymmetric inverted duplication chromosome. The loci involved in the inversion would be present in three copies, loci proximal to the inversion in four copies and those distal in two copies. There are a few reported cases of asymmetric supernumerary inverted duplication chromosome 15s

Figure 27. Paracentric inversion model of dicentric inverted duplication chromosome formation.



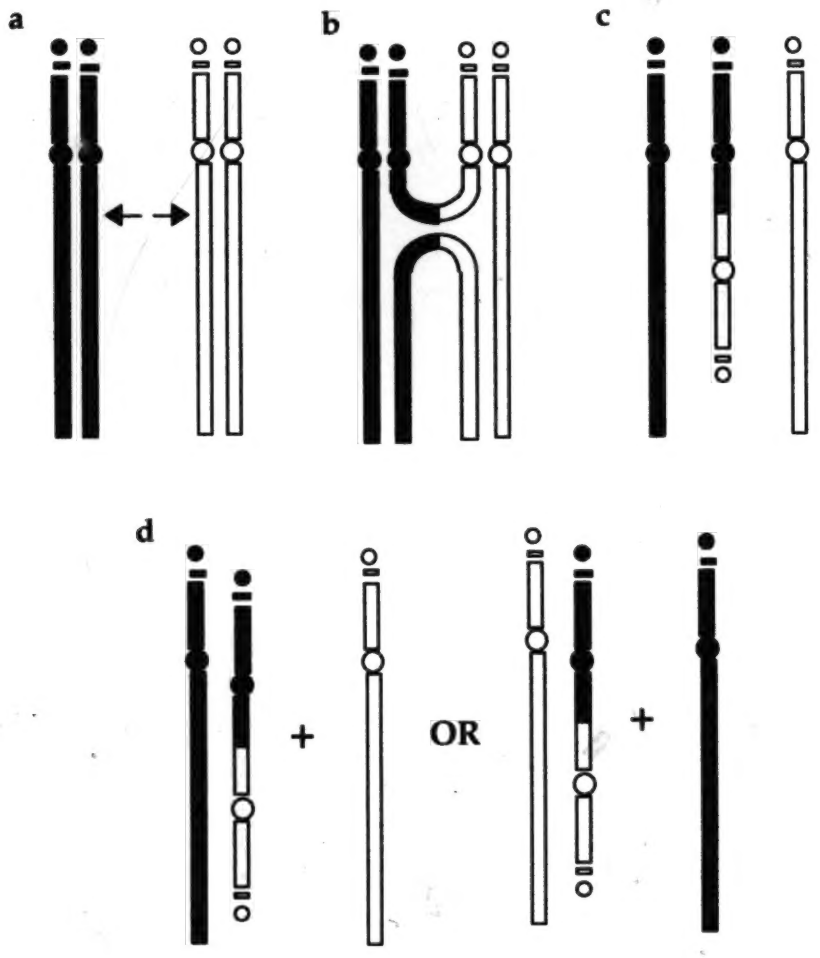
(Robinson et al., 1993b; Mignon et al., 1996; Leana-Cox et al., 1994- each of these report one inv dup 15) and CECs (3/12 studied in this thesis). Most of these supernumerary chromosomes however, are symmetrical, which would not support this model as a mechanism for formation of inverted duplication chromosomes unless the crossover event was limited to the proximal end of the inversion. Even if it were however, the inverted duplication chromosome would not truly be symmetric. It is important to note however, that determination of asymmetry is limited by the resolution of the probes used in the characterization of these chromosomes.

The second mechanism, proposed by Van Dyke et al (1977) and called a U-strand exchange, is depicted in Figure 28 and briefly described below. This model requires misalignment of repeats that are in the opposite orientation. During meiosis I of gametogenesis, non-sister chromatids break and then rejoin to form both an acentric and a dicentric fragment. The involvement of non-sister chromatids has been shown in Magenis et al (1988), Maraschio et al (1981), and Wisniewski et al (1979). Nondisjunction in meiosis I is followed by segregation in meiosis II, resulting in a gamete containing a supernumerary dicentric chromosome in addition to the normal complement. In this model, the breakpoint locations on the non-sister chromatids determine symmetry/asymmetry. This model would account for the observations of both symmetrical and asymmetrical CECs/ inv dup(15)s as well as the absence of chromosomal anomalies in the parents.

The vicinities of the CEC breakpoint regions are known to contain repeats (Halford et al., 1993a; Collins et al., 1995; Collins et al., 1997; Budarf, unpublished results) however, it is not known if these repeats are in an inverted orientation. To determine if this is the mechanism by which inverted duplication chromosomes form, the repeats need to be located and the breakpoints sequenced.

It is possible that sister chromatid exchange could alternatively occur. Maraschio et al (1981) reported one sister chromatid exchange event out of eight inv dup(15)s examined. The formation of this inv dup(15)

Figure 28. U-strand exchange model of dicentric inverted duplication chromosome formation modified from Schreck et al (1977). **a)** Two normal chromosome 22s with arrows representing abnormal breakage. **b)** Reunion of broken strands resulting in a U-type structure producing a dicentric structure and an acentric structure which is subsequently lost. **c)** Results of nondisjunction in meiosis I. **d)** Gametes produced after normal segregation in meiosis II.



may have occurred via pre-meiotic breakage of a single chromatid followed by replication and then fusion of the sticky ends to generate a dicentric chromosome. The result of an event such as this could generate only a symmetrical structure.

Mears (1995) proposed a model which he called the ARRC22 model.

Anchored
Repeat
Rearrangements of
Chromosome
22

This model was based on the literature regarding chromosome 22 which accounted for the formation of the multitudes of rearrangements (duplications, deletions, CECs) involving 22q11.2. Essentially this model proposes that repeats such as Alu sequences, LINES, minisatellites, and chromosome 22-specific low copy repeats are responsible for the misalignments which when accompanied by a recombination event could result in a rearrangement. With regards to formation of the CECs, like the U-strand exchange model, symmetry/asymmetry depends on which repeats align and where the recombination event occurs. Figure 29 is a depiction of how the ARRC22 model would generate the rearrangements in 22q11.2.

Ascertainment of the exact mechanism which is responsible for CEC formation must be preceded by further characterization of the repetitive sequences in this region and sequencing of the breakpoints themselves. Clustering of both the type I and type II CEC breakpoints presented here, in combination with the corresponding locations of the CATCH22 deletion breakpoints and the published data and unpublished suggestions that repeats exist in this region, supports a model in which repeat misalignment and subsequent recombination results in CEC formation.

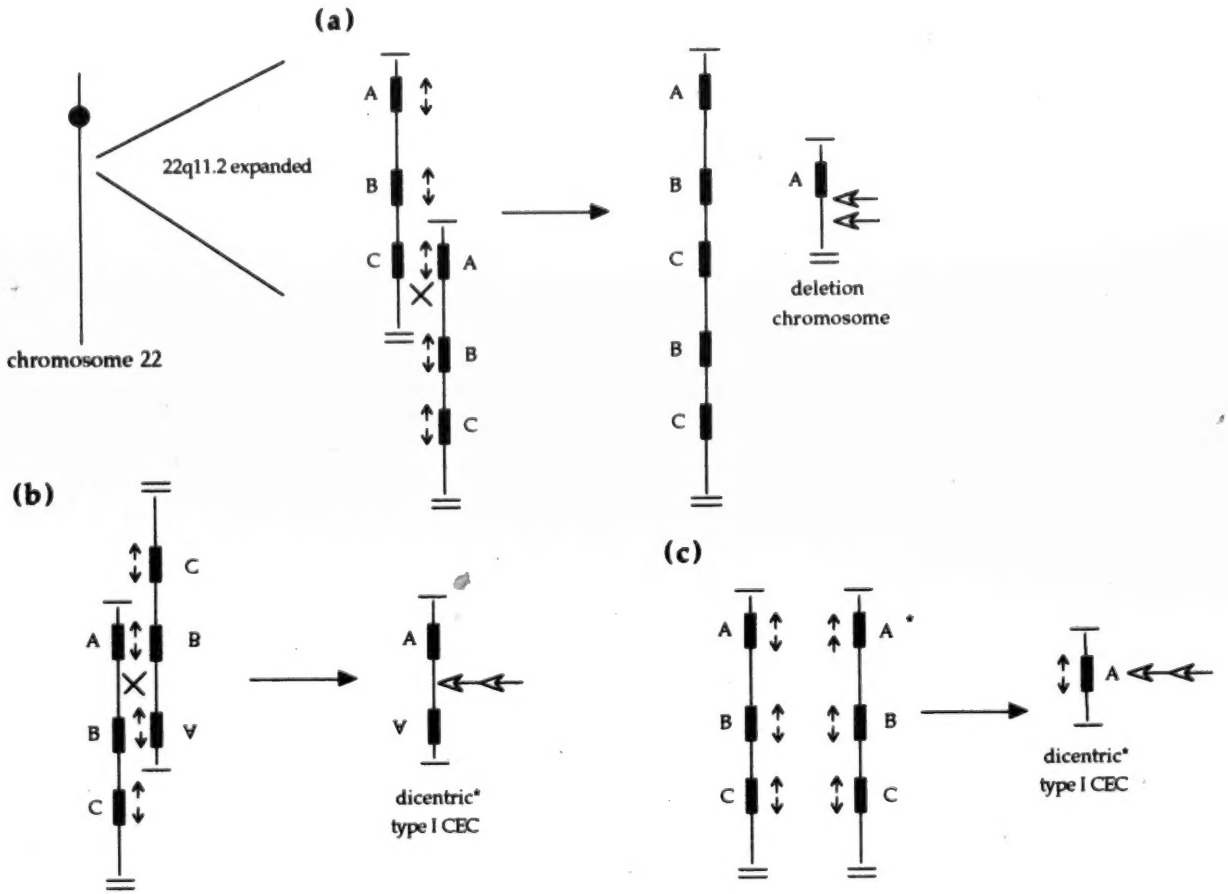
Figure 29. ARRC22 model.

A portion of 22q11.2 is expanded to demonstrate the possible role of repetitive sequences (black boxes) in the generation of the rearrangements common to this region. The boxes represent repetitive sequences described in Collins et al (1995, 1997) and the arrows represent the orientation of the repeats. The location of the crossover events are placed such that the observed breakpoints from Figure 24 are in proper relation to the repetitive sequences in Figure 23.

(a) Generation of the common CATCH22 deletion. The repeats in this case align directly. The open arrows represent the breakpoints which are found distal to the "A" repeat and distal to the "B"repeat. Misalignment and crossing over between repeat B and C on one chromosome and A and B on the other would generate the less common smaller CATCH22 deletion. There are numerous possibilities of misalignment and recombination.

(b) Generation of the dicentric CEC. Misalignment of repeats in a reverse orientation, followed by a recombination event could generate a dicentric structure. In the scenario represented, the "B" repeats are in an inverted orientation to the "A" repeats. A recombination event in the location above would generate a symmetrical type I CEC

(c) Generation of the dicentric CEC by misalignment of repeats polymorphic with respect to orientation. In order for the repeats in box "A" to pair, a loop must form which when followed by a recombination event, generates a dicentric chromosome.


(a)
22q11.2 expanded
chromosome 22
A
B
C
A
B
C
A
B
C
B
C
A
deletion
chromosome
(b)
C
A
B
B
C
A
dicentric*
type I CEC
(c)
A
B
C
A *
B
C
A
dicentric*
type I CEC

Supernumerary Ring Chromosomes

Supernumerary ring chromosomes represent a duplication of chromosomal material. The generation of these chromosomes is difficult to explain. The presence of a ring chromosome in an individual with only 46 chromosomes requires breakage of the ends of a chromosome and then fusion, and thus represents a deletion. The presence of a supernumerary ring chromosome however, requires more than one aberrant event. For example, formation of the ring itself from a normal chromosome requires the breakage-fusion event as well as non-disjunction of the remaining normal chromosome. The incidence of supernumerary ring chromosomes in prenatal studies is approximately 0.024% (Brøndum-Nielsen & Mikkelson 1995). Ring chromosomes account for only a small proportion of supernumerary marker chromosomes.

Supernumerary ring chromosomes have been associated with the presence of supernumerary inverted duplication chromosomes. For example, Adhvaryu et al (1995) described an unstable inv dup(15) chromosome in three generations of a family, all with normal phenotype. The dicentric marker chromosome was present in one member of each generation and a ring chromosome believed to be generated by the breakage of this marker chromosome was present in a second member of the third generation. M^{c}Ginniss et al (1992) present molecular data from a r(21) chromosome, consistent with formation by breakage of an isochromosome or Robertsonian translocation chromosome. Urioste et al (1994) describe a family demonstrating instability of a CEC in which cells from three family members representing two generations were examined. All three individuals were mosaic for the CEC, which had different morphologies in different cells. In summary, dicentric CECs, monocentric chromosomes, ring chromosomes, and fragments of different sizes were observed, indicating instability and suggesting that a ring chromosome can arise from breakage of a CEC.

These reports led Mears et al (1995) to speculate that the supernumerary ring chromosome 22 present in the grandfather of the

three generation family he studied could have arisen by the breakage of an unstable CEC and subsequent fusion in an earlier generation, resulting in a smaller duplication. Figure 30 depicts the formation of the ring chromosome of the grandfather from both a supernumerary CEC and a normal chromosome 22. Neither precursor can be ruled out with the information available.

The supernumerary r(22) chromosome from patient CM16 was found to contain a larger portion of proximal 22q than the r(22) of Mears et al (1995). The duplication of this ring chromosome extended even beyond the duplications found in the type II CECs. The location of the breakpoint compared to the type I and type II CEC breakpoints are shown in Figure 24 on page 117. The absence of a CEC larger than this r(22) suggests that either this ring is not derived from a marker chromosome, or that marker chromosomes larger than the type II CECs do exist but we have yet to find one. It is possible that such CECs do not exist due to a more severe phenotype caused by the larger duplication. There are reports of two individuals with larger interstitial duplications of 22q than the cases reported by Knoll et al (1995) and Reiss et al (1985) (Lindsay et al., 1995; Prasher et al., 1995). Although there are only the two cases of each for comparison, it appears as though the individuals with the larger interstitial duplications are more severely affected. Perhaps an individual with a CEC with two extra copies of the duplicated region may be more severely affected than the two reported individuals with larger interstitial duplications. Larger CECs could also be very rare due to the absence of another unstable region located more distal on the chromosome. Figure 23 on page 116 shows the location of similar repeats to the ones near the CEC breakpoints. Interestingly, just distal to the 22q breakpoint of the r(22) of patient CM16 are some low copy repeats (Collins et al., 1995; Collins et al., 1997). Although no definite conclusions can be drawn regarding their involvement in the formation of this ring chromosome, it is interesting that repetitive elements are found in this region as well.

If the supernumerary r(22) chromosomes do in fact arise from breakage of unstable CECs, one would expect to find some rings that have one copy of some of the probes in the duplicated region and two copies of

others, due to the nature of the structure of the CECs. Unfortunately, dosage analysis of patient CM16 could not be performed because of the mosaic state of the ring chromosome. Thus, I cannot conclusively determine the mechanism of formation of this supernumerary ring chromosome. In this case, fiber FISH would be useful in determining the number of copies of each locus present on the ring chromosome.

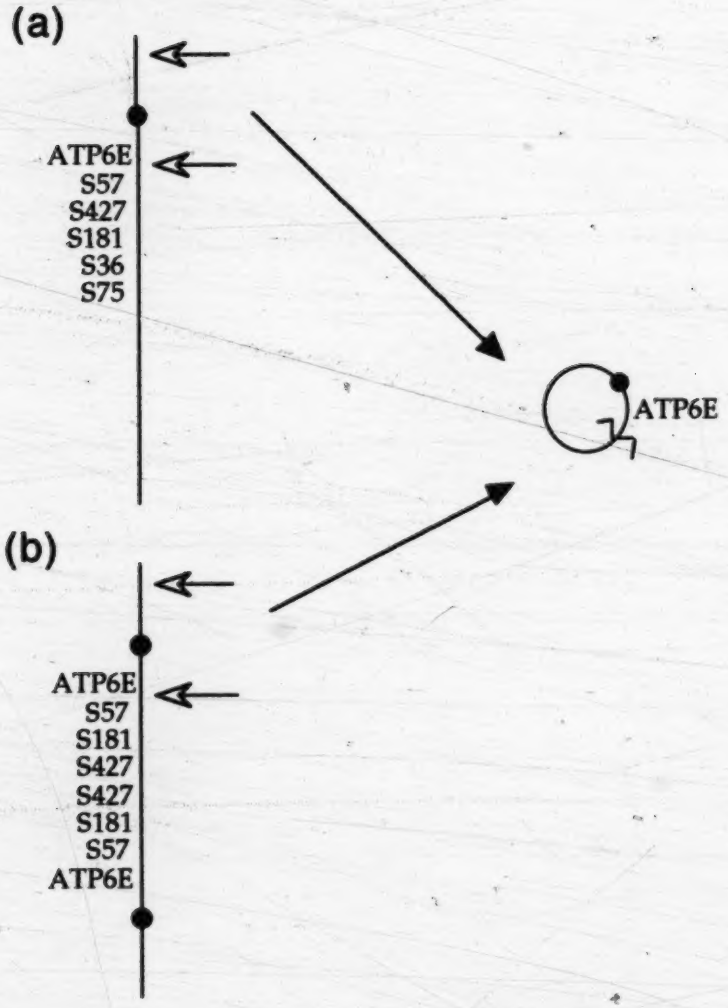
Despite the fact that the duplication of proximal 22q present in CM16 is larger than both the type I and type II CECs, the patient is relatively mildly affected. There are several explanations for this. The first is that the extent of duplication may not correlate with the severity of phenotype. As discussed in the genotype-phenotype correlations of type I and type II CEC duplications, the extent of duplication in this region does not appear to result in an increase in severity of phenotype. Another possible explanation for a mild phenotype is the presence of only three copies of the region rather than four, assuming the ring is not doubled. The presence of three copies of the CESCR can be consistent with a normal phenotype (Mears et al., 1995; Brøndum-Nielsen 1991), although the two reports of interstitial duplications suggests that individuals with interstitial duplications of the CESCR can have numerous CES features (Knoll et al., 1995; Reiss et al., 1985). For example, the individual reported by Reiss et al (1985) has very severe colobomas in addition to characteristic CES facial features. The individual in Knoll et al (1995) has TAPVR, a characteristic CES facies, and kidney and genital defects. Prediction of phenotypic severity based on the number of copies of the CESCR is thus not possible. The most likely explanation for the mild phenotype of CM16 is mosaicism. In general, mosaic individuals are more mildy affected than those who are not (Urioste et al., 1994; Cullen et al., 1993). Approximately 86% of lymphocytes in the initial peripheral blood sample possessed the supernumerary r(22) (Ohashi et al., 1993). Given the tremendous instability of ring chromosomes, this percentage likely fluctuated during development, and will likely fluctuate in the tissues of this patient in the future.

Figure 30. Possible mecharisms of formation of the r(22) of patient CM13 from Mears et al (1995).

(a) Formation by two breaks (arrows), one in the p-arm and the other proximal to D22S57 on the q-arm. Subsequent fusion will generate a monocentric ring chromsosome with extra copies of 22q loci up to and including ATP6E (Mears et al., 1995).

(b) Formation by breakage of a typical type I CEC and subsequent fusion. The result of this event would be a r(22) indistinguishable from that formed in (a).

The locations of the loci on the chromosome are not representative of their true position as they actually span a much shorter distance. Loci beginning with an "S" should be preceded by "D22". The jagged line on the ring chromosome indicates the point of fusion.


(a)
ATP6E
S57
S427
S181
S36
S75
ATP6E
(b)
ATP6E
S57
S181
S427
S427
S181
S57
ATP6E

CONCLUSIONS

Breakpoint Clustering

The purpose of this research was to localize breakpoints of the duplications associated with CES. The majority of the research focused on the CEC which is the most common form of the duplication. The CECs are divided into two types based on the extent of the duplication. The smaller type I CECs possess two copies of the CESCR. Both breakpoints involved in the formation of these chromosomes occur within a 450-650kb interval flanked by loci D22S427 and D22S36 (see Figure 3 on page 21). The larger type II CECs also possess two copies of the CESCR as well as one or two copies of the neighboring CATCH22 deletion region (see Figure 12 on page 78). The type IIa CECs are asymmetrical, possessing one extra copy of the CATCH22 deletion region. One of the breakpoints of each type IIa CEC is located in the type I interval. The other breakpoints have been localized to an interval flanked by CRKL and D22S112. The type IIb CEC is symmetrical, possessing two copies of the CATCH22 deletion region with both breakpoints occurring in the CRKL-D22S112 interval. Despite the fact that individuals with a type II CEC possess one or two extra copies of the CATCH22 deletion region, their phenotype is no more severe than individuals with a type I CEC.

The clustering of breakpoints of the CECs when considered alone is unlikely to be mere coincidence, even though only a limited number of cases have been characterized. This clustering is even more striking when comparison is made to the CATCH22 deletion breakpoint intervals. Figure 24 on page 117 depicts these intervals. Detection of low copy repetitive sequence in these regions provides a possible explanation for the occurrence of rearrangement "hotspots".

Identification of the type II breakpoint intervals enabled the testing of the hypothesis of ring chromosome formation put forth by Mears et al (1995) and Mears (1995). The 22q breakpoint involved in the formation of the supernumerary r(22) of patient CM16 occurs in an interval distal to the type II CEC interval. Thus, either this ring did not form by the breakage of

a CEC, or it did, and a larger class of CEC from which it was derived exists but have not yet been characterized. Unfortunately, conclusive determination of the mechanism of formation was not possible.

Clinical Implication

One major difference between the CECs and the inv dup(15)s is the absence of a correlation between the size of the chromosome and the phenotype in the case of the CECs. Both subgroups of the smaller class of the inv dup(15) chromosomes have a relatively mild, or no resultant clinical phenotype. The larger class, which are duplicated for the PWS/AS region, demonstrate a recognizable phenotypic spectrum. Thus for the inv dup(15)s, characterization of the chromosome has some predictive value. Those with duplications extending up to but not including locus D15S9 have little or no clinical consequence. On the other hand, to date no phenotypic differentiation is possible for the type I and type II CECs. Classification of the CECs into these two groups thus has no predictive value. There are however, reports of supernumerary bisatellited marker chromosomes of chromosome 22 origin with no clinical phenotype (Blennow et al., 1994; Blennow et al., 1995; Gravholt & Friedrich 1995; Daniel et al., 1994; Rauch et al., 1992; Verschraegen-Spae et al., 1993). Thus we believe that a smaller class of inverted duplication 22 chromosome does exist with breakpoints proximal to the current CESCR. Proximal to locus D22S50 in the pericentromeric region are low copy repeats similar to those found near the breakpoints of the type I and type II CECs (Figure 23 on page 116). Definition of the breakpoints of these small markers may enable some form of predictive testing of prenatal samples possessing these extra chromosomes as well as narrowing the CESCR.

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APPENDIX I
Solutions and Reagents

Bacterial/Yeast culture medium

Luria-Bertani (LB) medium

10g bacto tryptone
5g yeast extract pH 7.5
10g sodium chloride
1 Litre

SD Medium

8g yeast nitrogen base without amino acids
20g glucose
55mg adenine sulphate
55mg L-tyrosine
930 ml
After autoclaving, add 70ml 20% casamino acids (trp^- ura^-)

DNA Preparation

Blood Lysis Buffer

8% DTAB (40g/500ml total solution) (dodecyltrimethylammonium bromide $C_{15}H_{34}NBr$ - SIGMA B8638)
50mM EDTA (pH 8)
100mM Tris (pH 8)
1.5M NaCl

CTAB Solution

5% CTAB (2.5g/50ml total solution) (mixed alkyltrimethylammonium bromide $C_{14}H_{29}N(CH_3)_3Br$ - SIGMA M-7635)

Southern Hybridization

OLB (Oligo Labelling Buffer) for random priming

100ul 1M Tris (pH 7.5)
12.5ul 1M $MgCl_2$
12.5ul each 10mM dATP, dGTP, dTTP
250ul HEPES (pH 6.6)
150ul 90 A_{260} units/ml oligos (random primers -Pharmacia)
(to 50U vial, add 556ml ddH_2O to give 90U/ml)

50X Denhardts

2% w/v BSA powder (Fraction V SIGMA)	5g/500ml
2% w/v polyvinyl pyrrolidone	5g/500ml
2% w/v ficoll 400 (Pharmacia)	5g/500ml

Prehybridization solution (a modification of Church & Gilbert's)

350ml 10% SDS
132ml 1M $NaPO_4$ (pH 6.5-6.8)
50ml 50X Denhardts
500ul 1M EDTA

FISH

10X dNTP mixture

0.2mM each dCTP, dGTP, dTTP
0.1mM dATP
0.1mM biotin-14-dATP
500mM Tris-HCl (pH 7.8)
50mM magnesium chloride
100mM B-mercaptoethanol
100ug/ml nuclease free BSA

10X enzyme mix

0.5 units/ ul DNA Polymerase I
0.0075 units/ ul Dnase I
50mM Tris-HCl (pH 7.5)
5mM magnesium acetate
1mM B-mercaptoethanol
0.1mM phenylmethylsulfonyl fluoride (PMSF)
50% (v/v) glycerol
100ug/ ml nuclease-free BSA